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THE NEUTRALIZATION OF ANTIPNEUMOCOCCUS IMMUNE BODIES BY INFECTED EXUDATES AND SERA.

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That sterile filtered inflammatory exudates have the power to modify the course of infection was noted long ago. The chief discussion concerning the nature of this phenomenon has been carried on by Bail and his associates and by those who have contraverted their views. Bail[1] gave to the hypothetical substances existing in pathological exudates, which alter the course of infection, the name "aggressins." He thought that they were secreted by the bacteria during their growth in the animal body and acted by inhibiting or by neutralizing the defensive mechanisms of the body. According to this investigator it is to these substances that the bacteria owe their power to invade the body tissues and therefore it is upon their ability to form them that the property of virulence depends.

Wassermann and Citron[2] have opposed the view of Bail that these so called aggressins represent substances set free by the bacteria during their struggle against the protective agencies of the body, and believe that they represent merely bacterial substances which may go into solution either within the body or during growth or autolysis *in vitro*, and that these substances act by fixing the humoral immune bodies and so rendering them ineffective; that the mode of action of these substances therefore does not differ from that of dead bacteria. It is not german to the present discussion to consider the large amount of evidence which has been brought forward to support the contending views, the chief purpose of the present paper being to record the demonstration of large amounts of substances which neutralize immunity principles in the blood and exudates of animals infected with pneumococci, and to indicate the importance of these substances in the specific therapy of acute lobar pneumonia.

The part which these substances play in experimental pneumococcus infections, especially their effect on phagocytosis, has been studied by Hoke,[3] Rose-

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[1] Bail, O., *Arch. Hyg.*, 1905, lii, 272.

[2] Wassermann, A., and Citron, J., *Deutsch. med. Woch.*, 1905, xxxi, 1101.

[3] Hoke, E., *Wien. klin. Woch.*, 1905, xviii, 348.

now,[4] Tschistowich and Jurewich,[5] Zade,[6] and Nunokawa.[7] Tschistowich and Jurewich have made the observation that thoroughly washing virulent, non-phagocytable pneumococci in salt solution is sufficient to render them phagocytable. Rosenow, as well as Tschistowich and Jurewich, has also observed that treating non-virulent, phagocytable pneumococci with the washings from, or extracts of virulent pneumococci is sufficient to render the former non-phagocytable and therefore virulent, and he has found that this property is retained even after washing in salt solution. This writer has given to the hypothetical substances which may be extracted from virulent pneumococci the name "virulin," and Tschistowich and Jurewich have given to the substances which they have obtained by somewhat similar methods the name "anti-phagin."

It is evident that these observations, especially those of Rosenow, would render necessary an entirely different conception of the phenomenon from that held by Wassermann and Citron.

In a considerable number of experiments, however, I have been unable to confirm the observations of Rosenow that non-virulent pneumococci may absorb and fix something derived from virulent pneumococci which renders the former virulent, and for the present, therefore, I am inclined to accept the explanation offered by Wassermann and Citron, especially since this conception is sufficient to explain all the following observations.

The first observations on which this communication is based were made on the fluid removed from the chests of persons suffering from empyema. The fluid from these cases was examined for its content in pneumococcus immune bodies as tested by agglutination and protection. Similar tests of the patient's blood showed that it possessed well marked protective and agglutinative properties, and we were therefore surprised when we found that the empyema fluid possessed no such powers. A probable explanation seemed to be that, although the immune bodies were originally present in the exudate, they had been absorbed by the bacteria present, just as they may be from immune blood serum when bacteria are added *in vitro*. It occurred to us, however, to test this empyema fluid after removal of bacteria, to determine whether or not there might be present soluble substances,

[4] Rosenow, E. C., *J. Infect. Dis.*, 1907, iv, 285.

[5] Tschistowich, N., and Jurewich, Y., *Ann. Inst. Pasteur*, 1908, xxii, 611.

[6] Zade, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 81.

[7] Nunokawa, K., *Z. Immunitätsforsch., Orig.*, 1909, iii, 172.

which would fix or divert the immune substances contained in immune serum. The following is a protocol of one experiment.

Case 1.—E. R.; age 19 years. Acute lobar pneumonia followed by empyema due to Pneumococcus Type I. 200 cc. of thick pus were removed at operation. A portion of the fluid was centrifugalized at high speed for 30 minutes, the supernatant fluid was removed to a fresh centrifuge tube and again centrifugalized for 1 hour, and finally diluted with an equal quantity of isotonic saline solution and again centrifugalized for 30 minutes. The perfectly clear fluid as examined microscopically contained no organisms.

Agglutination Tests								
0.4cc Serum I	+	0.4cc empyema fluid				30 min at 37° C.	0.1cc Cult. I	2 hrs. 37°–18 hrs. ice
" " (1:10)		"	"	"			"	++
" " (1:20)		"	"	"			"	+
" " (1:40)		"	"	"			"	–
" " (1:50)		"	"	"			"	–
" " (1:100)		"	"	"			"	–
" " (1:200)		"	"	"			"	–
" " (1:10)		NaCl					"	++
" " (1:20)		"					"	++
" " (1:40)		"					"	++
" " (1:50)		"					"	++
" " (1:100)		"					"	++
" " (1:200)		"					"	++
———		Empyema fluid undiluted					"	–
———		"	"	1:20			"	–
———		"	"	1:40			"	–
———		"	"	1:100			"	–

TEXT-FIG. 1. Protocol of an experiment showing the inhibiting action of empyema fluid on the agglutination of pneumococci by immune serum.

This fluid was then tested for its power to cause agglutination of Type I pneumococci and also for its power to inhibit the agglutination of pneumococci by Type I immune serum. The results are given in Text-fig. 1.

The fluid was then tested for its power to inhibit the protective action of immune serum against infection with Type I pneumococci as tested in mice. To guard against the possibility that the fluid, centrifugalized as noted above, might contain an occasional pneumococcus which might interfere with the result, it was heated for 30 minutes at 56°C. Cultures made from this fluid were sterile.[8] Text-fig. 2 gives the results of this experiment. They show in a striking way that empyema fluids may contain large amounts of soluble substances which inhibit the action of immune serum.

Protection Tests					
Culture dilution cc.	0.2cc. i.h.s. + 0.2cc. empyema fluid	0.2cc. i.h.s. 0.2cc. n.h.s.	0.2cc. NaCl + 0.2cc. empyema fluid	Culture control	Empyema fluid alone cc.
0.1	D. 20 hrs.	S.	D. 30 hrs.	—	0.5 S
0.01	" 21 "	"	" 16 "	—	0.3 "
0.001	" 30 "	"	" 38 "	—	0.2 "
0.0001	—	—	" 30 "	—	0.1 "
0.00001	—	—	" 30 "	D. 30 hrs.	
0.0000001	—	—	" 30 "	" 30 "	

TEXT-FIG. 2. Protocol of an experiment showing the inhibiting action of empyema fluid on the protection of mice by immune serum.

Similar tests have been carried out with a series of these empyema exudates removed by aspiration or at operation. The results have not been so striking in all the cases examined as those shown in the above protocol, though some degree of inhibition has been present in all infected cases. Several sterile serous fluids aspirated from the chest of pneumonia patients, however, have not exhibited this phenomenon. The degree of inhibiting action is apparently dependent upon the degree of infection and the time the infection has lasted before aspiration is performed.

These observations indicate why it is that infections in the partially immunized animal tend to be focal and why, when an animal is infected with organisms of slight virulence, the infection tends to remain localized. It is probable that as soon as bacteria begin to grow in tissue spaces these inhibiting substances appear in the in-

[8] In making the tests with mixtures of serum and fluid, the mixtures were allowed to incubate for 30 minutes at 37°C. before injection.

flammatory exudate, and when the fluid is not readily absorbed the substances accumulate in large amounts, so that finally, as in empyema, it is practically impossible to produce a focal immunity reaction until the focus is opened and the fluid, with its content of neutralizing substance, is removed by drainage, when the bacteria remaining are no longer protected from the natural or artificial defensive mechanisms of the body and so may be overcome. This conception agrees in the main with the view held by Bail and others, though the application of the theory has previously been made rather to the problems of virulence and infection than to those of recovery. The observations previously mentioned also indicate that favorable results can hardly be expected from the treatment of these focal infections with immune serum, either administered intravenously or injected directly into the focus itself unless the pathological exudate has previously been removed. We have made one attempt to treat a patient suffering from empyema by the direct injection of immune serum into the cavity, but without apparent effect. These observations offer the explanation for the failure. In the treatment of focal infections with immune serum, without drainage, it would be necessary to inject sufficient serum to neutralize all the inhibiting substances present, as well as the amount necessary to prevent the harmful activities of the bacteria themselves.

Our next problem was to discover whether or not the inhibiting substances appear in the blood as a result of septicemia. This was first investigated by inoculating rabbits with very large injections of pneumococcus and testing the blood removed during the height of infection for the presence of these substances. To show that this action is due to soluble substances, and not to the bacteria present, the bacteria have been removed from the serum by filtration before testing. The results of one of these experiments are given in the following protocol.

Rabbit 1.—Weight 1,300 gm. July 11, 1916, 12 noon. Inoculated intraperitoneally with 1 cc. of peritoneal exudate of a rabbit previously infected with Type II pneumococci. 5 p.m. Blood culture shows innumerable numbers of pneumococci.

July 12, 10 a.m. Animal very sick. Blood removed by heart puncture. Serum removed from clot and passed through a Berkefeld filter. Culture of filtered blood sterile. The filtered blood was tested for its power to inhibit the

agglutinating action of immune horse serum, Type II. Table I shows the results obtained.

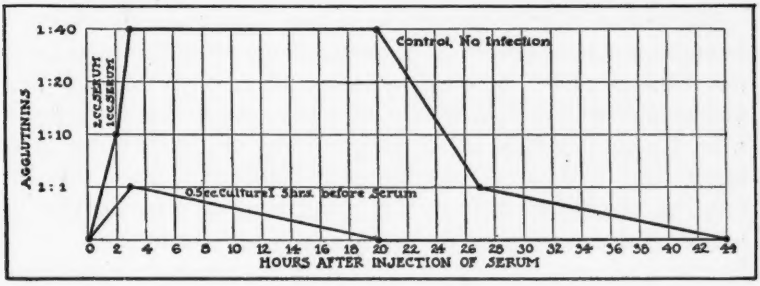
TABLE I.

Agglutination.		Results after 2 hrs. at 37°C. and 18 hrs. on ice.
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of filtered serum	After 30 minutes at 37°C. 0.1 cc. of Culture II was added to each tube.	−
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of filtered serum		−
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of filtered serum		−
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of filtered serum		−
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of salt solution		++
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of salt solution		++
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of salt solution		++
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of salt solution		++
0.4 cc. of Immune Serum II (1:400) + 0.4 cc. of salt solution		+
0.8 cc. of salt solution		0

The results of this and other similar experiments show that specific inhibiting substances such as those which are present in pathological exudates may also be present in the blood when an animal is suffering from a severe septicemia.

Another method which has been used for testing the presence of inhibiting substances in the blood is the following. A rabbit is infected with pneumococci and after the infection has reached its height immune serum is injected intravenously. At the same time, and as a control, a normal rabbit receives the same amount of immune serum intravenously. Within a few minutes and at varying periods following the injection of the serum, samples of blood are removed from both rabbits and tested for their content in antibodies. For this purpose agglutination is employed. If no neutralization of antibodies occurs, it is evident that the content of the blood in agglu-

tinins immediately following the injection should be the same as though the immune serum had been diluted *in vitro* with a quantity of fluid equal to the blood contained in the rabbit, and that by making repeated tests a curve showing the disappearance of the immune bodies by destruction or excretion may be constructed. As a matter of fact, numerous observations in normal rabbits have shown that when the rabbit's blood is tested within a few minutes following the injection of immune serum, its content in agglutinins is about that to be expected when the probable volume of blood in the rabbit and the consequent dilution of the immune serum is calculated. On the other hand, when a similar injection is made into an infected animal,



TEXT-FIG. 3. Curves showing the agglutinating power of the serum of normal and infected rabbits following the injection of immune horse serum.

the agglutinating power of the serum obtained from it is much less than that calculated from the probable dilution; indeed, agglutinating power may be entirely absent. Moreover, when the agglutinating power is present, though lower than that of the serum of the normal rabbit, and curves are made to show the disappearance of the agglutinating power, it is found that the agglutinins disappear much more rapidly from the serum of the infected rabbit than they do from the serum of the uninfected rabbit. Text-fig. 3 shows in a graphic manner the results obtained in one of these experiments.

In these experiments the possibility cannot be excluded that the fixation or neutralization of antibodies is due to the presence of bacteria circulating in the rabbit's blood, but previous observations make it

improbable that the entire phenomenon can be due to this. It seems probable that the neutralization is due to a considerable extent to the presence of soluble inhibiting substances.

This last method of study is directly applicable to patients, and a study of this kind in patients is of importance since it is difficult to produce in animals pneumococcus infections which last over a period of a week or longer, such as those which occur in man. Moreover, it was hoped that this study would offer indications for proper dosage of serum and might even be applicable in the treatment of the individual case. In a series of cases, therefore, the serum has been tested for its content in agglutinating antibodies both before and following the administration of immune serum.

The method of procedure was as follows: Samples of the patient's serum were obtained before any immune serum was administered and also 5 minutes following the first dose. Where more than one dose was administered (and successive doses have usually been given with 6 to 8 hour intervals) other samples were obtained immediately before and 5 minutes following each subsequent dose. Finally, following the last dose, in certain cases, samples were obtained at varying periods to observe the persistence of agglutinins in the blood. The samples from each patient were kept on ice until all had been obtained and they were then tested on the same day and with the same technique for the presence of agglutinins. The agglutination tests were made by the macroscopic method. In each of a series of small test-tubes was placed 0.9 cc. of the serum, or of the diluted serum. To each of the tubes was then added 0.1 cc. of an 18 hour broth culture of pneumococcus of the type to which the infection was due and corresponding to the serum which had been injected. The results were read by transmitted light after 2 hours at 37°C. and again after the tubes had remained on ice over night.

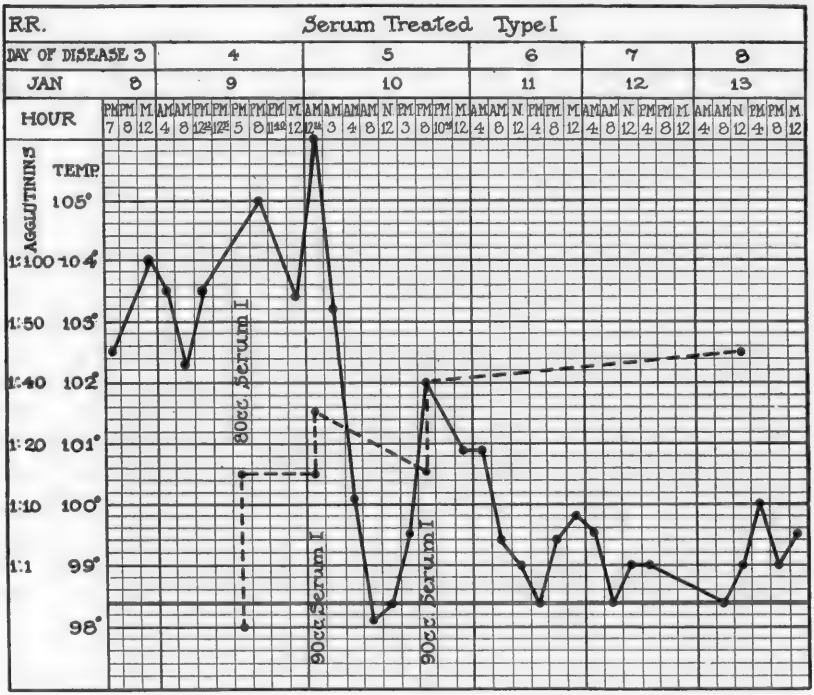
Employing these methods, agglutinin curves have been made from 30 cases suffering from Type I infection and receiving serum, 9 cases suffering from Type II infection, and also serum treated, 7 cases with Type II infection, who received no serum, 4 of Type III, not serum treated, 2 of Type IV not serum treated, and 1 case due to Type IV infection who, through a mistake in diagnosis, received several doses of Type I serum, making a total of 53 cases.

Cases Due to Type I Infection Treated with Serum.

Of these cases, 3 were treated on the 2nd day, 5 on the 3rd day, 5 on the 4th day, 4 on the 5th day, 9 on the 6th day, 3 on the 7th day, and 1 on the 9th day. All but two of the cases recovered. The charts and protocols of several cases, which illustrate the relation of agglutinin titer to the clinical course and to the temperature curve, are given below.

Case 1.—R. R., student; age 22 years. This patient was admitted January 8, 1917 at 7 p.m. suffering from pneumonia involving the left lower lobe. The onset had been quite typical with chill, 48 hours before admission. He was moderately sick; temperature 102.5°F., pulse 115, respirations 30. The leukocytes numbered 31,000 and the blood culture was positive, the plates showing one colony per cc. of blood. The sputum was bloody; a small amount was at once inoculated into a mouse. The following morning tests made of the growth in the peritoneal cavity of the mouse showed that the patient was suffering from an infection with Type I pneumococci. 12.17 p.m. The intravenous injection of antipneumococcus serum was commenced. Although the serum was given slowly, after he had received about 35 cc. he had some signs of serum intoxication, suffusion of the face, respiratory difficulty, and he vomited several times. The administration of serum was therefore at once discontinued. No tests were made of the agglutinating power of his serum before or after this treatment. The patient's condition did not materially change during the afternoon and at 5 p.m. serum was again administered; this time 80 cc. were given without any untoward symptoms. A sample of blood was taken just before and another one 5 minutes following the administration of the serum. When tested later, it was found that the blood before administering the serum contained no agglutinins for pneumococcus; the sample of blood taken following the administration of serum agglutinated Type I pneumococcus in a dilution of 1:15. This represents a concentration of antibodies fully equal to that which might be expected, taking into consideration the titer of the serum injected and the probable volume of the patient's blood. The patient's condition did not materially improve after this injection, so that another dose of 90 cc. of serum was administered at 12 midnight. A specimen of blood which was obtained just before this injection showed that the agglutinating power had not diminished during the time intervening since the preceding dose, and the specimen of blood taken 5 minutes after the serum was injected showed an increased concentration of agglutinins, so that now agglutination occurred with a 1:30 dilution of serum. Immediately following this injection the temperature rose to 106°F. and he had a shaking chill. The temperature then began to fall, being only 98.1°F. at 8 a.m. With this fall in temperature, the patient's condition markedly improved. During the day, the temperature again rose slowly, without, however, any other unfavorable features. As the temperature at 8 p.m. was

102°F., it was decided to administer another dose of serum, and 90 cc. were given, without any reaction. The test of the patient's serum obtained before this treatment showed that the agglutinins had fallen slightly, agglutination occurring in a dilution of 1: 15, but after the treatment the titer again rose to 1: 40. Following this treatment the patient made a good recovery.

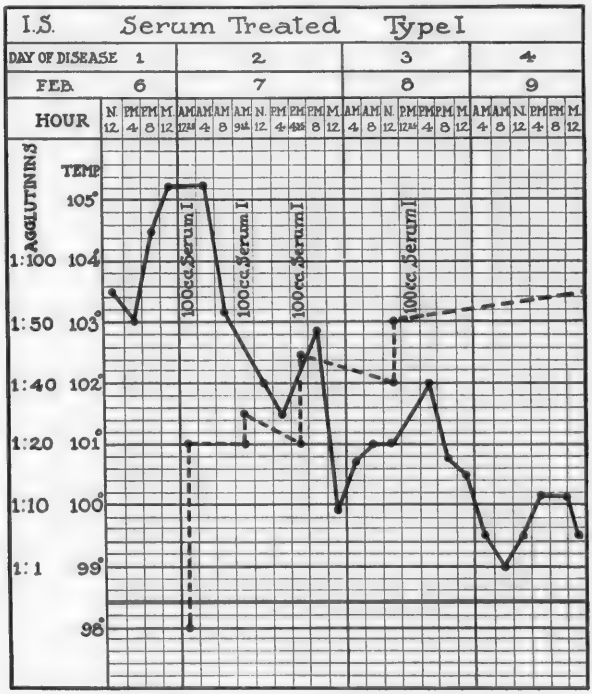


——— Temperature curve.
- - - - - - Curve of agglutinin titer.

TEXT-FIG. 4. Chart showing the curve of the agglutinin titer and the temperature curve of R. R.

In this case, therefore, which was mild or of only moderate severity, treated early in the disease, the administration of immune serum was followed by a satisfactory concentration of antibodies in the blood and there was no evidence of fixation or neutralization of the injected immune substances. This is graphically shown in Text-fig. 4.

Case 2.—I. S., tailor; age 32 years. This patient was admitted about 8 hours following the initial chill. There were definite signs of involvement of both lower lobes and he presented all the characteristic features of acute lobar pneumonia. His temperature on admission was 103.5°F., pulse 120, respirations 48. He appeared seriously sick; the blood culture taken on admission was positive. The diagnosis of the type of infecting organism was made by inoculation of sputum



TEXT-FIG. 5. Chart showing the curve of the agglutinin titer and the temperature curve of I. S.

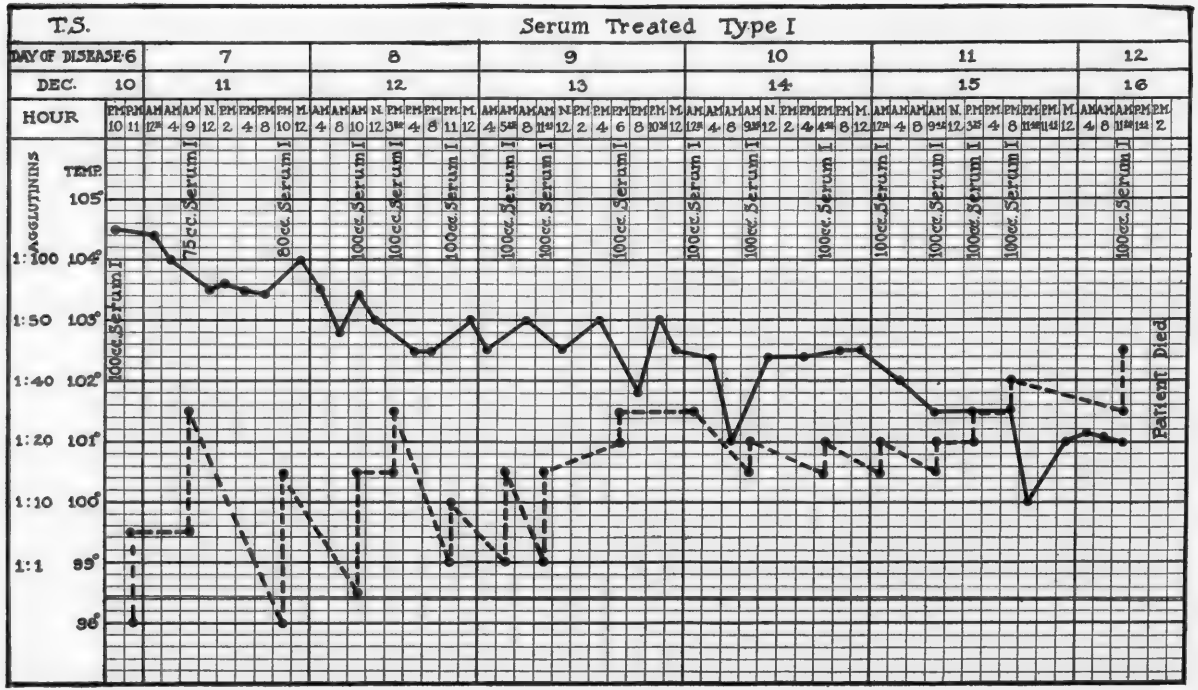
into a mouse, and at 12 midnight, 12 hours after admission, he was given his first treatment. As the curves presented in Text-fig. 5 show, the first dose of serum imparted to the blood a well marked power of agglutination, and each subsequent dose was followed by an increase of this property. With the increase of agglutinins in the blood there occurred an improvement in the patient's condition, the temperature fell, and he finally recovered completely.

The two examples given above illustrate the effects of serum treatment in the cases due to Type I infection, when the serum is given early and in large amounts, and when the infection has not reached too high a grade before the treatments are commenced. Since in twenty-eight out of the thirty cases recovery followed the administration of the serum, we did not have great opportunity to study cases of this type in which the serum was not effective. In all the cases treated with this type of serum agglutinins could be demonstrated in the patient's blood 2 to 3 minutes after the administration of 75 to 100 cc. of serum. The agglutinating power varied somewhat, though in most cases it occurred with a dilution of 1:10 or more. The refinements of the method are not sufficient to justify our calculating in each case the probable dilution and the consequent probable loss in agglutinins in the short interval elapsing before the first tests were made. In general, where the administration of subsequent doses of the serum has not led to a prompt increase of the agglutinins in the blood above the previous level the fall of temperature has been longer delayed and more serum has been required than in the cases in which a regular step-like rise took place. In comparing the temperature and agglutination curves in these cases it has been necessary to keep in mind the fact that the temperature alone does not offer a safe and sure criterion for judging the patient's condition and therefore for the effectiveness of the serum. In view of this fact it has been surprising to see the considerable uniformity with which the temperature and agglutination curves run in opposite directions. With rise of agglutinating power the temperature curve falls.

Of more importance than the immediate rise in agglutinins following the first dose is probably the persistence of the agglutinins in the blood during the subsequent 8 to 10 hours elapsing before the following dose of serum is given. In only five of the cases did a decrease during this period occur. In three of the cases the loss occurred only following the first dose. Following the subsequent doses the concentration reached a high level and persisted. The data in these three cases is not sufficient to enable us to state categorically that this loss indicated a greater severity of infection, though taken in connection with our other observations this seems probable. Two of these three cases were treated on the 4th day and one on the 6th. They

required two, three, and four doses of serum respectively and all made good recoveries following the serum treatment. In one of the other cases in which the agglutinating power disappeared before the following dose was given, the concentration of immune bodies following the first three doses was such that agglutination did not occur with dilutions greater than 1: 5, and following the first two doses this power disappeared completely before the subsequent dose was given. It was only after numerous doses had been given that the concentration of agglutinins reached any considerable level and persisted. Altogether this patient required eleven doses of serum given over 7 days. Treatment was commenced in this patient on the 2nd day, but it was not pushed with great vigor at the start, the first dose being 80 cc., with 18 hours elapsing before the administration of the second dose of 70 cc., and 12 hours again elapsing before the administration of the third dose of 80 cc. This case suggested very strongly the inadvisability of inactive treatment at the start. This patient ultimately recovered and there occurred no extension of the lesion to other lobes, but the temperature remained high for 10 days and he was very ill. Finally, the last case in which the agglutinins disappeared between subsequent doses and in which there occurred difficulty in causing a persistent concentration of immune bodies in the patient's blood by the administration of immune serum was one of the two cases which ended fatally. The curves taken from the record of this case are shown in Text-fig. 6. It is apparent from the curves that it was not until treatment had been continued for 3 days, and nine doses had been given, that a persistent concentration of immune bodies at a high level was attained. Even following this there was a constant tendency for the concentration of immune bodies in the serum to fall, rather than to rise.

It should be noted that the treatment in this patient was commenced only on the 6th day, and 11 and 13 hours elapsed between the first and second, and the second and third doses, respectively. He was desperately ill on admission; temperature 104.5°F., pulse 136, and the blood culture showed an extremely high grade of infection, over 300 colonies per cc. In spite of this he lived until the 12th day. It seems that in this case the serum prolonged life. The infection and intoxication, however, at the start were so great that, although the



TEXT-FIG. 6. Chart showing the curve of the agglutinin titer and the temperature curve of T. S.

infection could be kept down, the intoxication could not be recovered from. In this instance it is likely, judging from the experimental observations, that the presence of large amounts of soluble inhibiting substances in the blood prevented the action of the immune serum. It is probable that in such cases very late in the disease these substances may be so large in amount that no practical amount of immune substances can neutralize them. If these conceptions are correct, the importance of giving very large doses of immune serum at the beginning of treatment is apparent.

In the other fatal case, persistent high concentration of immune bodies in the patient's blood was obtained without difficulty. Nevertheless, the patient's condition did not improve and repeated doses of serum were administered. Type I pneumococci had been obtained from the sputum, and the blood culture showed 47 colonies per cc. of the same organisms. Treatment was commenced on the 6th day and the patient died on the 10th day. The pathological changes were extensive in both lungs. The autopsy showed a very widespread tuberculous involvement of both upper lobes and the upper portion of the lower lobe on each side. At the base of one lung, however, was a small area of complete consolidation, differing in appearance from the remainder of the tuberculous lung. This proved on study to be a typical acute diffuse pneumonic process and from it Type I pneumococci were cultivated. We have here an instance in which the serum was apparently effective against the specific infection, but death occurred on account of factors associated with the primary and extensive tuberculosis.

A further interesting case in this connection was one due to Type IV infection. Owing to a mistake in the early determination of the type of infection the patient received several doses of Type I serum before the mistake was discovered. In this patient, although he was quite ill, the administration of the serum caused a prompt appearance of agglutinins in the blood and this increased with the subsequent doses, without any material fall.

These studies of agglutination curves in the cases of Type I infection, however, while instructive and suggestive, do not after all give definite proof that the effect of immune serum is limited by the presence of soluble substances in the blood. When they are con-

sidered, however, in the light of the observations on the Type II cases which follow, the evidence becomes much more suggestive.

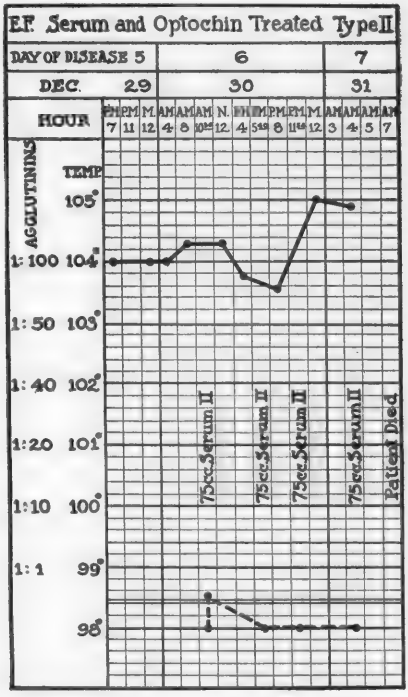
Cases Due to Type II Infection.

The reader should be reminded at the outset that it has been impossible to produce a serum against Type II pneumococci which is as active either *in vitro* or *in vivo* as is the serum against Type I pneumococci. Whereas the horse serum against Type I infection is of such a strength that 0.2 cc. will regularly protect a mouse against 0.1 cc. of virulent culture, it has been impossible to produce an immune Type II serum of any greater activity than that 0.2 cc. will protect a mouse against 0.01 cc. of culture. Moreover, the active Type I sera usually cause agglutination of homologous organisms in dilutions of 1:400 or over; the Type II sera usually cause agglutination in dilutions no greater than 1:200. It should also be noted that the capsule formation of Type II pneumococci is more highly developed than is that of Type I pneumococci. Dochez and Avery[9] have pointed out that production of precipitable substances in the blood and urine of infected animals apparently bears some relationship to this property of capsule development, the Type III organisms, which possess large capsules, forming most of this substance, the Type II organisms, which have smaller capsules, producing less, and the Type I organisms, which have small capsules, producing still less. While it is not certain that the substances in the infected animals which give rise to fixation of antibodies are identical with those concerned in the precipitation phenomenon, it seems likely that this is the case.

Studies of the agglutinin content of the blood were made in nine cases of Type II infection which received Type II serum. Of these patients, four recovered and five died. Of the patients who recovered, in two treatment was commenced on the 3rd day, in one on the 4th day, and in one on the 5th day. Of the fatal cases, treatment was commenced in one on the 3rd day, in one on the 5th day, and in the others on the 6th day. In all the four cases which recovered, a satisfactory and persistent concentration of agglutinins in the blood appeared. In one of the fatal cases, practically no agglutinins ap-

[9] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

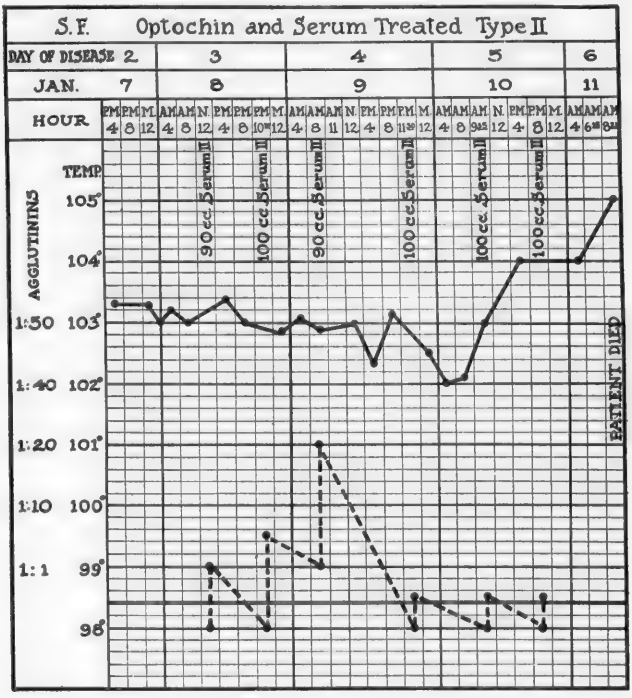
peared in the blood in spite of four doses of serum; in one a satisfactory concentration appeared only after three doses, and then disappeared; in another a satisfactory concentration was obtained only on the 9th day, after five doses of serum; in another, while a satisfactory concentration of agglutinins was obtained on the day



TEXT-FIG. 7. Chart showing the curve of the agglutinin titer and the temperature curve of E. F.

treatment was commenced, the 6th day, the patient was at that time suffering from meningitis, from which he died 2 days later. In the remaining case only one dose of serum was given, 2 hours before death; no agglutinating power appeared in the blood. Text-figs. 7 and 8 show graphically the results of observations made in two of the fatal cases.

Case 1.—E. F., porter; age 46 years. This patient was admitted on the 5th day of the disease with an extensive lung involvement and very severe septicemia, the blood cultures showing 1,000 colonies per cc. His condition was very serious; temperature 104°F., pulse 104, respirations 64, and, in spite of four doses of serum within 24 hours, the patient died. It will be noted that immediately following the administration of 75 cc. of serum, the undiluted blood showed power



TEXT-FIG. 8. Chart showing the curve of the agglutinin titer and the temperature curve of S. F.

of agglutination, but this property had disappeared within 7 hours, and following the subsequent doses the blood showed no agglutinating power whatever. It is evident, therefore, that in this severely infected case the immune bodies in the serum disappeared from the blood as fast as they were administered.

Case 2.—S. F., clerk; age 31 years. This patient, though admitted early in the disease, was extremely ill; temperature 103.3°F., pulse 160, respirations 56. The blood culture showed 400 colonies per cc. and the following morning, before the

first dose of serum was given, the culture showed 1,600 colonies per cc. Following the first two doses of serum there occurred an immediate appearance of agglutinins in the blood in low dilutions, which, however, disappeared or became minimum in amount before the succeeding doses. In spite of the extreme grade of blood infection, the number of organisms present in the blood diminished following these two doses, the cultures on the morning of the 4th day showing only 20 colonies per cc. There was a satisfactory increase in agglutinins following the third dose, but it will be noted that 15 hours were allowed to elapse between this dose and the succeeding one, and during this time the agglutinins had entirely disappeared and the subsequent doses produced little or no effect on the agglutinin content (Text-fig. 8). The patient died on the 6th day.

Five patients suffering from Type II infection who received no serum were also studied to observe the appearance of agglutinins in the blood. All these recovered. In four of these instances at the end of the disease there developed well marked power of agglutination; in one of them agglutination in the serum obtained on the 10th day occurred in a dilution of 1: 100. In the fifth no agglutinins appeared in the blood, though this was not studied later than the 12th day. This patient received optochin, as did, however, several of the cases in which agglutinins developed.

DISCUSSION.

Neufeld and Haendel,[10] Dochez,[11] and others have shown that specific immune substances usually appear in the blood during recovery from lobar pneumonia. This is shown by an increase in protective power of the blood for mice against homologous infection. Clough[12] has made similar observations and he and others have also noted that in certain instances the protective power is accompanied by the power of inducing *in vitro* phagocytosis of virulent homologous pneumococci which are not phagocytable in normal serum. It would seem, therefore, that bacteriotropins represent one form of immune body playing a part in this protective phenomenon. Bull[13] has brought forward experimental evidence which indicates strongly that the phenomenon of agglutination is of great importance in the

[10] Neufeld, F., and Haendel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 166.
[11] Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.
[12] Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 295.
[13] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

action of immune serum in pneumococcus infection. It is possible that several different antibodies or phenomena take part in the mechanism of pneumococcus humoral immunity. The observations I have mentioned, as well as unpublished observations made in this laboratory, indicate strongly that natural recovery in pneumonia is associated with the development of humoral immunity and probably occurs because of this development. In the individual case, however, the factors which determine recovery or death cannot be stated so simply. In mild cases probably a very slight grade of humoral immunity may be sufficient to prevent progress of the disease, a grade of immunity which can be detected with difficulty by our present means. In other instances the reaction required on the part of the body may be very great and the immunity phenomena exhibited by the serum when tested outside the body may be very vigorous and marked.

The phenomenon of agglutination offers one ready means for testing the degree of humoral immunity. It is, however, not the only one and it is unsafe to judge of the immunological effectiveness of a serum solely by its agglutinating strength. The protective power and agglutinating power of immune horse serum, however, tend to run parallel. Consequently, the study of agglutinating power of the blood of patients, such as has been made in the present instance, must be of considerable value in indicating the presence or absence of humoral immunity. If recovery in pneumonia is due to the development of humoral immunity, the study of its appearance during recovery and especially of its appearance following treatment with immune serum, should be of significance. In commencing the study it was thought that the method might be employed to graduate the dosage of immune serum in the treatment of the individual case. If recovery is due to the appearance of immune bodies in the blood, the ideal serum treatment would be such that sufficient serum be administered to produce the required concentration of immune bodies and no more. It soon became apparent, however, that such a method, testing the blood before and after the administration of each dose, involved so much time and labor that it would not be of practical value. It has seemed, however, that the repeated tests of the serum in a series of cases, as has been done here, give us considerable

knowledge of the mode of action of the serum and offer valuable suggestions for the routine dosage and mode of application of the serum. The studies have further indicated strongly that during infection not only must sufficient immune substances be added to bring about a concentration sufficient to sensitize all the bacteria, produce their agglutination, opsonification, etc., but in addition there must be a sufficient amount administered to neutralize any soluble substances present in the serum which have the property of neutralizing and fixing the immune substances. It is realized that the occurrence of these soluble, fixing substances in the blood of infected patients has not been directly demonstrated. The experimental observations in animals previously described, however, make it altogether probable that these substances are present in severe infections. It must be admitted that in most instances where there was failure of immune substances to appear in the blood, or where the immune bodies disappeared very rapidly following their administration, bacteriemia was shown to be present before the first dose of serum was administered. In several cases, however, the blood infection could not be demonstrated after the first dose, and nevertheless, rapid disappearance of the immune bodies occurred following the subsequent doses. In one instance in which the rapid disappearance of immune bodies occurred, the blood cultures taken both before and after the administration of serum were sterile. However, it seems likely that in all cases when fixation of immune bodies occurs, blood infection has at some time been present, though the possibility that the fixing substances may, in certain instances, arise entirely in local foci cannot be excluded.

The nature of the substances bringing about the fixation can at present only be conjectured. The demonstration, however, by Dochez and Avery[9] of substances giving rise to precipitates in the blood and urine of infected patients makes it probable that the same substances are responsible for the phenomenon of fixation that we have studied. They have apparently shown that these substances may be excreted or formed by the bacteria during their growth, and it is also probable that substances contained in the bacteria and set free during their dissolution may give rise to the same phenomenon.

The observations made in this study have a practical bearing on the

question of the therapeutic administration of immune serum. The amount of serum necessary to be given does not depend merely on the weight of the patient and therefore on the consequent dilution of the serum in the body. It is also not entirely dependent on the degree of infection present. If the patient is treated early before large amounts of the soluble substance are present, a moderate amount of serum may be sufficient, even though the grade of blood infection may be considerable. On the other hand, if the infection has continued for a considerable time, and large amounts of soluble, fixing substance are present in the blood, the amount of serum required may be very large. It is therefore evident that it is important that the patient be treated as early as possible and before large amounts of these fixing substances are formed. Moreover, the importance of treating very actively at the start in order that all these fixing substances may be at once neutralized and the progress of the infection immediately and entirely overcome is apparent. It is therefore our plan at present to treat all patients with Type I infection with large initial doses, and to repeat the treatment every 6 to 8 hours as long as may be necessary. It is possible that the Type II serum is less effective than Type I serum not only because its concentration of immune bodies is less than that of Type I serum, but also because the power of pneumococci of this type to produce fixing substances is more highly developed than is that of pneumococci of Type I.

CONCLUSIONS.

1. In empyema fluids resulting from infection with pneumococci there are present large amounts of soluble substances which have the property of neutralizing pneumococcus antibodies.

2. Similar substances are found in the blood of infected rabbits.

3. When immune serum is injected into infected rabbits the immune substances disappear very quickly, and therefore are prevented from activity in overcoming the infection.

4. When immune serum is administered to patients severely infected with pneumococci, the immune bodies may also disappear very rapidly, and this disappearance is probably associated with the presence of such soluble substances in the blood.

5. The serum only becomes effective when these substances are neutralized.

6. The study of agglutination curves is of value in showing why in certain instances favorable results have not followed the use of immune serum.

7. It is important that in severely infected patients the serum be administered early in the disease and that the initial dosage be large.

THE ELABORATION OF SPECIFIC SOLUBLE SUBSTANCE BY PNEUMOCOCCUS DURING GROWTH.

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From the study of bacterial infections in man and the lower animals evidence has been accumulated that pathogenic organisms do not produce harmful effects from their mere physical presence. The general reaction or toxemia of infection is differentiated from the local process which in many instances is the only tangible expression of bacterial invasion. Substances of a harmful nature seem to pass out from the bacteria and through the circulating medium of the animal to injure cells and organs at a distance from the site of infection. An explanation of the nature and mode of action of these substances has been one of the great problems in the study of infection. Certain bacteria, such as diphtheria, tetanus, and others, when grown in artificial media form a soluble toxin whose action when injected into animals differs in no way from that manifested when the same bacteria grow in living tissue. The majority of pathogenic bacteria are not known to form these soluble toxins during their life processes. The capacity of the latter to intoxicate has been explained by their setting free upon death a toxin which during life is retained within the cell body. Some investigators hold the view that intoxication by these bacteria arises in the body of the infected animal from the splitting of the bacterial protein into toxic degradation products. These explanations of bacterial intoxication are not so satisfactory nor so well substantiated as are the facts concerning infection with bacteria which produce known soluble toxins.

Pneumococcus is a highly pathogenic microorganism which is not known to secrete a soluble toxin, and whose harmful effects are supposed to be due either to the setting free of intracellular toxins or

to the formation on disintegration of toxic split products. In the present paper it is shown that this organism during the early stages of its growth forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the circulating blood, whence it passes through the kidneys into the urine. We have not as yet been able to demonstrate with certainty that this substance is responsible for the intoxication that accompanies lobar pneumonia.

Formation of a Soluble Substance in Culture Media.

In 1897 Kraus[1] demonstrated for the first time the presence of specific precipitable substances in the germ-free culture filtrates of certain bacterial species. This work was subsequently extended by other observers so that it is now known that a large number of bacteria give rise to these substances in the media in which they grow. The precipitin reaction obtained with these substances is strictly specific and occurs only when an homologous immune serum is used. These culture fluids have been studied after the bacteria have grown in them for 24 hours or more and their presence has been supposed to be due to the passing into solution of the bacterial substance upon disintegration of the cells. The same precipitable substances may be demonstrated in the bacteria-free salt solution or distilled water extracts of organisms grown on solid media.

Neufeld[2] has shown that solutions of pneumococcus obtained by the addition of small amounts of bile to bouillon cultures produce a specific precipitate in the presence of immune rabbit serum. Wadsworth[3] has obtained similar results not only with bile solutions but also with filtered salt solution extracts of pneumococcus. Panichi[4] demonstrated the presence of a specific precipitable substance in the filtrate of bouillon cultures of pneumococcus.

The fact which we wish to emphasize in this study is that pneumococcus from the time it starts to grow elaborates in the medium of its environment a specific substance of bacterial origin in considerable amounts and that the early presence of this soluble substance is not attributable to the death and subsequent disintegration of the bacterial cell, but represents the extrusion into the medium of bacterial substance during the life processes of the organism.

[1] Kraus, R., *Wien. klin. Woch.*, 1897, x, 736.
[2] Neufeld, F., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 54.
[3] Wadsworth, A., *J. Med. Research*, 1903–04, x, 228.
[4] Panichi, L., *Centr. Bakteriol., Ite Abt., Orig.*, 1907, xliii, 188.

TABLE I.

Rate of Growth of Culture.

No. of hrs.	Colonies per cc.
0	810,000
2	1,170,000
4	26,000,000
6	245,000,000
8	297,000,000
12	382,000,000
24	No growth from 0.0001 cc.
48	" " " 0.1 "

Titration of Precipitin Reaction in Culture Fluid.

Dilution.	After 0 hrs.	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 12 hrs.	After 24 hrs.	After 48 hrs.
1:1	−*	±	+++					
1:5	−	−	++					
1:10	−	−	±	+++	+++	+++	+++	+++
1:15	−	−	±	+++	+++	+++	+++	+++
1:20	−	−	±	+++	+++	+++	+++	+++
1:25	−	−	±	++	++	+++	+++	+++
1:30	−	−	±	+	++	++	++	++
1:40	−	−	−	+	+	++	++	+
1:50	−	−	−	±	+	+	+	+
1:60	−	−	−	±	±	±	±	±
1:80	−	−	−	±	±	±	±	±
1:100	−	−	−	−	−	±	±	±
1:120	−	−	−	−	−	±	±	±
1:160	−	−	−	−	−	−	−	±
1:200	−	−	−	−	−	−	−	±
1:240	−	−	−	−	−	−	−	±
Control.	−	−	−	−	−	−	−	−

* − indicates no reaction; ±, faint trace; +, visible precipitation; ++, flocculation; +++, heavy flocculation.

In the following experiment a flask of bouillon was incubated with a small amount of an early, rapidly growing culture of pneumococcus. A young culture was chosen in order to avoid the occurrence of bacterial lag, during which some cell death occurs. At varying intervals during the growth of the culture, fractions were withdrawn from the flask, freed from bacteria, and the cell-free fluid was tested for the

presence of precipitable substances. A bacterial count was made of each specimen in order to determine that the culture was growing at a maximum rate and that little or no cell disintegration had occurred at the time when this substance was already present in considerable amounts. The quantity of precipitable substance present in a given specimen was determined by ascertaining the maximum dilution of the cell-free fluid at which precipitation occurred on the addition of homologous antipneumococcus serum.

A protocol in which Type III pneumococcus was studied is given, since this organism forms a large amount of soluble substance, whereas Type II and Type I form lesser amounts in the order named.

The bacterial counts in the experiment given (Table I) show that the cultures grew at a maximum rate for about 12 hours. Chesney[5] has shown in an elaborate study that during this period the bacteria increase in geometric progression and that the curve of generation time may be plotted as an ascending straight line. From this the deduction may be drawn that during the first 12 hours little or no cell death occurs. Examination of the precipitin reaction with the bacteria-free filtrates of specimens removed from the culture during the first 12 hours of growth reveals the fact that the bacterial substance passes into solution in the culture medium in easily demonstrable amounts during this time. This would seem to indicate that this soluble substance is not the result of bacterial disintegration but represents an actual extrusion of the cell substance into the medium during the life processes of the organism.

Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Infected Rabbits.

The demonstration that pneumococcus during its growth in fluid media gives rise to a soluble substance suggested the likelihood that the same substance might be detected in the body fluids of experimentally infected animals. In order to test this assumption, a rabbit was injected intraperitoneally with 1 cc. of the blood of a rabbit infected with pneumococcus. At varying intervals after infection specimens of blood were collected from the heart, and the serum,

[5] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

freed from cells, was passed through a Berkefeld filter in order to remove the organisms that had reached the blood stream. The bacteria-free serum was then tested for the presence of precipitable substances by the addition of homologous immune serum. The urine of these rabbits was also tested to find out whether the soluble bacterial substance passed through the kidneys and could be demonstrated by the precipitin reaction in the animal's urine. In Table II an example is given in which the rabbit had been infected with a Type II pneumococcus.

TABLE II.

Pneumococcus Precipitin Reaction in the Blood Serum of a Rabbit Infected with Pneumococcus Type II.

Time.	Before infection.		2 hrs. after infection.		4 hrs. after infection.		6 hrs. after infection.		8 hrs. after infection.	
Type serum....	I	II	I	II	I	II	I	II	I	II
Result.....	−*	−	−	±	−	+±	−	++	−	++

* − indicates negative; ±, faint trace; ++, marked flocculation.

Tests of rabbit urine cannot be made at regular intervals because of the failure of the animal to void frequently. Specimens at the end of 24 hours, however, showed a marked precipitate when mixed with the serum corresponding in type with the organism with which the animal was infected.

The experiment given in Table II demonstrates the fact that within a short period of time after intraperitoneal injection of a rabbit with pneumococcus there is present in the filtered blood serum a specifically reacting bacterial substance of pneumococcus origin. This substance readily passes from the blood through the kidneys into the urine and can there be demonstrated in considerable concentration.

Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Patients Suffering from Lobar Pneumonia.

The fact that the pneumococcus forms a readily soluble substance during growth in artificial media and in the body fluids of animals

experimentally infected makes it not unreasonable to assume that the same substance is formed by pneumococcus during the course of natural infection in human beings. In order to find out whether or not this is so, the blood sera and urine of a large number of patients suffering from pneumonia due to pneumococcus of Types I, II, and III were studied for the presence of this soluble material. Specimens of serum were obtained at varying intervals during the disease and the urine was examined frequently throughout the course of the infection and during convalescence. If the precipitin reaction was not positive with the whole urine, a method of concentrating the urine was employed. It has been found that the soluble substance to which the pneumococcus gives rise is precipitated by alcohol and after precipitation is again readily soluble in water. In order to concentrate the precipitable substance in urine to 25 cc. or more of the 24 hour specimen a few drops of acetic acid are added and the urine is then boiled down to a volume of 5 cc., filtered through paper to remove any precipitate of albumin that may occur, and the filtrate added to eight to ten volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifugalization and dried to remove the excess of alcohol and the residue extracted with 2 or 3 cc. of salt solution which redissolves the specific substance. Any insoluble material is removed by centrifuging and the clear salt solution extract used in the precipitin test. Results of this study are presented in Table III.

In Table III are presented the studies on the presence of the specifically precipitable substance in the blood serum during life of a number of patients suffering from lobar pneumonia. Almost all the patients studied showed a strongly positive precipitin reaction in the urine and were chosen for the purpose of finding out whether at a time when the substance was being excreted in largest amounts it could also be demonstrated in the circulating blood. In all, 25 cases were examined, of which 10 were due to infection with Type I pneumococcus, 11 with Type II, and 4 with Type III. Of the Type I infections, none gave a positive precipitin reaction in the serum, although in all but one the urine was positive at the time the tests were made and in three instances the reaction in the urine was heavy, indicating the excretion of the soluble substance in considerable

TABLE III.

Pneumococcus Precipitin Reaction in the Blood Serum during Lobar Pneumonia.

Case No.	Blood test.		Blood culture.	Urine.	Serum treatment.	Result of disease.	Remarks.
	Day of disease.	Result.					
Pneumococcus Type I infection.							
2,821	6	−	−	++	+	Recovered.	Total, 10 cases; 2 died.
2,816	8, 9, 10, 11, 12	−	+	++	+	Died.	
2,901	5, 7, 9	−	+	++	+	"	
2,968	6, 7	−	+	+	+	Recovered.	
2,936	7, 9	−	−	±	+	"	
2,815	4	−	−	+	+	"	
2,824	3	−	−	±	+	"	
2,883	5, 6, 7	−	−	−	+	"	
2,858	3, 4, 5	−	−	+	+	"	
2,891	3	−	−	+	+	"	
Pneumococcus Type II infection.							
2,885	3, 4, 6	−	−	+	+	Recovered.	
2,845	6	++	+	++	+	Died.	
2,868	4	−	−	−	−	Recovered.	
	7			+			
2,829	1	−	−	−	−	"	
2,879	2	−	+	−	+	"	
	7			+			
2,892	5, 6	+	+	++	−	Died.	
2,834	6, 7, 9, 10, 11	−	+	++	+	"	
2,922	4, 5, 6	−	−3	+	+	Recovered.	
			+7				
3,006	2, 3	−	−	++	−	Died.	Blood culture positive on 6th day.
3,031	3, 5, 7	+	+	++	+	"	
2,869	3, 4, 5	+	±	+	+	"	
Pneumococcus Type III infection.							
2,898	5, 6, 7	++	+	++	−	Died.	Total, 4 cases.
2,947	6, 10, 11	±	−	+	−	Recovered.	
2,797		+	−	+	−	Died.	
2,783	7	+	+	+	−	"	

quantity. The failure to demonstrate the substance in blood in Type I pneumonias may be partly attributable to the fact that all these cases were treated with Type I antipneumococcus serum, which is known to cause the disappearance of the substance from the urine in many cases during treatment. It has also been shown by *in vitro* experiments that Pneumococcus Type I forms less of the soluble substance than organisms of Types II and III. The sera of 11 cases of Type II pneumonia were studied. The urine reaction was positive in 10 of these, while the precipitin reaction in the blood was positive in 4 instances. Of these 11 cases 7 were treated with Type II serum. Among the 7 serum treated cases, 3 showed a positive precipitin reaction in the blood. Of the 4 cases not treated with serum, only 1 gave a positive blood test. A positive blood culture was obtained in 7 of the 11 cases studied. Of the 4 cases with a positive precipitin test in the serum, all showed a positive blood culture, while of the 7 cases with a negative precipitin test in the serum, 3 had a positive blood culture. All 4 patients showing a positive precipitin reaction in the blood serum died, whereas of the 7 with a negative serum test, 2 died and 5 recovered.

4 cases of pneumonia due to Pneumococcus Type III were studied. All gave a positive precipitin test in the blood serum. Blood cultures were positive in 2 instances and the precipitin reaction in the urine was positive in all. The infection was fatal in 3 of the 4 patients.

In Table IV are presented the results of the examination of the urine for the precipitable pneumococcus substance in 88 cases of pneumonia due to the fixed types of pneumococcus I, II, and III. Of these 88 cases, 35 were Type I, 28 were Type II, 8 were Type II (atypical), and 17 were Type III. Repeated tests of the urine were made during the course of the disease from within 12 hours after onset in one instance to the 58th day in another. Of the 35 cases due to Type I infection, 20 were positive and 15 negative. A positive blood culture occurred in 13 of the 35 cases. Among the 13 cases with positive blood culture, 9 showed a positive urine reaction and 4 gave a negative result. Of the 20 cases with positive urine reaction, 2 died; of the 14 negative cases all recovered. All these instances of Type I infection were serum treated. The administration of serum

TABLE IV.

Pneumococcus Precipitin Reaction in the Urine during Lobar Pneumonia.

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type I infection.								
				days				
2,816	++	6	6	6	+	+	Died.	
2,936	+	6	6	18	−	+	Recovered.	
2,968	++	5	5	14	+	+	"	
2,858	+	3	4	1	−	+	"	
2,996	+	3	3	15	−	+	"	
2,891	+	3	3	5	−	+	"	
2,965	+	6	6	16	−	−	"	
2,952	+	2	5	3	−	+	"	
2,804	+	6	6	26	−	+	"	
2,955	++	6	6	26	+	+	"	
2,925	+	1	1	1	+	+	"	
2,908	+	5	13	5	−	+	"	
2,924	+	5	5	22	−	+	"	
2,913	+	5	5	1	−	+	"	
2,945	+	5	5	29	+	+	"	
2,906	+	2	2	31	+	+	"	Delayed resolution.
2,852	+	4	4	2	+	+	"	
2,949	+	8	11	24	−	+	"	
2,901	+	5	5	4	+	+	Died.	
2,917	−	6	−	−	−	+	Recovered.	
2,814	−	2	−	−	−	+	"	
2,944	−	5	−	−	−	+	"	
2,874	−	3	−	−	+	+	"	
2,815	−	4	−	−	−	+	"	
2,824	−	3	−	−	−	+	"	
2,821	−	6	−	−	−	+	"	
2,984	−	7	−	−	−	+	"	
3,020	−	3	−	−	+	+	"	
3,043	−	3	−	−	−	+	"	
3,011	−	5	−	−	+	+	"	
2,883	−	4	−	−	−	+	"	
2,954	−	4	−	−	−	+	"	
2,880	−	3	−	−	+	+	"	
3,019	+	4	8	42	+	+	" (empyema).	
3,033	−	2	−	−	−	+	Recovered.	

TABLE IV—*Continued.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type II infection.								
				days				
2,869	++	2	2	2	+	+	Died.	
2,922	+	3	3	40	+	+	Recovered.	
3,006	+	2	2	8	−	−	Died.	
2,885	+	2	2	2	−	+	Recovered.	
2,845	++	5	5	2	+	+	Died.	
2,868	+	4	7	3	−	−	Recovered.	
2,899	−	1	−	−	−	−	"	
2,879	−	2	7	1	+	+	"	
2,892	++	5	5	2	+	−	Died.	
2,834	++	5	5	5	+	+	"	
2,991	+	1	2	58	−	−	Recovered.	
2,854	−	3	5	1	−	−	"	
3,003	+	7	7	13	−	−	"	
2,827	++	3	3	1	+	−	Died.	
2,746	+	3	6	9	+	−	"	
2,881	+	6	6	2	−	−	Recovered.	
2,786	−	6	−	−	−	−	"	
2,896	−	7	−	−	−	−	"	
2,926	−	4	−	−	−	−	"	
2,897	+	3	4	1	−	+	"	
2,886	+	3	3	6	−	−	"	
2,890	+	3	3	4	+	+	Died.	
2,971	+	7	14	10	−	−	Recovered.	
2,825	++	1	1	5	+	−	Died.	
3,031	+	3	3	5	+	+	"	
3,047	+	3	3	?	−	−	Recovered.	
2,937	−	1	−	−	−	−	"	
2,934	−	4	−	−	−	−	"	
Pneumococcus Type II (atypical) infection.								
2,861a	−	3	−	−	+	−	Recovered.	
2,878x	−	4	−	−	−	−	"	
2,822b	−	5	−	−	−	−	"	
2,831x	+	4	4	3	−	+	"	
2,864b	+	8	8	2	+	−	Died.	
2,960x	−	1	−	−	−	−	"	
2,963b	+	4	7	13	−	−	Recovered.	
2,935b	−	1	−	−	−	−	"	

TABLE IV—*Concluded.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type III infection.								
				days				
2,838	−	3	−	−	−	−	Recovered.	
2,889	−	7	−	−	−	−	"	
2,797	++	2	2	5	−	−	Died.	
2,898	++	5	5	3	+	−	"	
2,947	++	5	5	30	−	−	Recovered (empyema).	
2,812	+	2	5	1	+	−	Died.	
2,783	+	7	7	1	+	−	"	
2,919	+	5	6	4	−	−	"	
2,800	+	3	3	3	+	−	"	
2,849	+	3	3	2	+	−	"	
2,927	+	2	6	4	−	−	Recovered.	
2,973	+	3	10	4	−	−	"	
2,911	+	2	2	13	−	−	"	
2,837	−	1	−	−	−	−	"	
2,972	+	1	6	2	−	−	"	
2,485	−	6	−	−	+	−	Died.	
2,918	−	2	−	−	−	−	Recovered.	

Summary of Urine Reaction.

Type.	Total No. of cases.			Per cent positive.	Positive blood culture.	Positive urine reaction.		Negative urine reaction.		Fatal cases showing urine reaction.	
	Examined.	Positive.	Negative.			With blood culture positive.	With blood culture negative.	With blood culture positive.	With blood culture negative.	Positive.	Negative.
				per cent							
I	35	20	15	57.1	13	9	11	4	11	2	0
II	28	20	8	71.4	11	10	10	1	7	10	0
II (atypical)	8	3	5	37.5	2	1	2	1	4	1	1
III	17	12	5	70.5	6	5	7	1	4	7	1
	88	55	33	62.5	32	25	30	7	26	20	2
IV	10	0	10	0	2	0	0	2	8	0	2
Cases of respiratory disease due to organisms not pneumococcus.	14	0	14	0	2	0	0	2	12	0	2

in Type I pneumonia often results in the temporary disappearance of the substance from the urine. Upon cessation of treatment the soluble substance may reappear in the urine.

Of 28 cases of Type II infection, 20 gave positive precipitin reaction in the urine, and 8 were negative. A positive blood culture occurred in 11 of the 28 cases. Among the 11 cases with positive blood culture, 10 gave a positive urine reaction and 1 a negative result. Of the 20 cases with a positive urine test, 10 died; of 8 negative cases all recovered.

Among 8 cases of infection with atypical Type II pneumococcus, 3 gave a positive precipitin reaction in the urine and 5 a negative reaction. Inasmuch as a normal Antipneumococcus Serum Type II was used in determining the presence of soluble substance in the urine of individuals infected with atypical Type II pneumococcus, a lower percentage of positive urine reactions should be expected in this series, since the precipitin titer of normal Type II serum is low for these atypical organisms.

17 cases of pneumonia due to infection with Pneumococcus Type III were studied. Pneumococcus precipitinogen was demonstrated in the urine of 12 of these instances, and was absent in 5. A positive blood culture was obtained in 6 of the 17 cases. Of the 6 cases having pneumococcus septicemia, 5 showed a positive precipitin reaction in the urine. 7 of the 12 cases giving a positive urine test died, while 4 of the 5 negative cases recovered.

A summary of 88 cases of pneumonia due to the fixed types of pneumococcus shows that the soluble substance of pneumococcus origin was demonstrable in the urine of 55 (62.5 per cent) of these patients at some stage of the disease and in 39 instances was positive on the first examination. Among the 55 cases with a positive precipitin reaction in the urine, 20 had a fatal outcome, giving a mortality of 36.4 per cent, and of the 33 cases with a negative urine test, 2 died; a mortality of 6 per cent. In addition to the 88 individuals suffering from pneumonia due to the fixed Types I, II, and III, 10 cases of Type IV pneumonia and 14 cases of respiratory disease due to other organisms were studied for the presence of a precipitin reaction in the urine. Each urine was tested with standard Antipneumococcus Sera Types I, II, and III. In no instance was a posi-

tive reaction obtained at any stage of the disease, a fact which establishes beyond doubt the specificity of the reaction.

In addition to the presence of the soluble substance of pneumococcus origin in the blood and urine, it has also been found in other body fluids. In certain cases it can be readily demonstrated in pleural fluids and pericardial exudates and in the spinal fluid of pneumococcus meningitis.

Certain facts have been ascertained concerning the chemical characteristics of this substance. The specific substance is not destroyed by boiling. It is readily soluble in water and is precipitable in acetone, alcohol, and ether, after which it may be easily redissolved in water. It is precipitated by colloidal iron, and does not dialyze through parchment. The immunological reactions of the substance are not affected by proteolytic digestion with trypsin and it is not split by urease. The determination of total nitrogen and nitrogen partition on the active substance obtained by repeated precipitation with acetone and alcohol shows the substance to be of protein nature or to be associated with protein.

One of the chief points of interest in the discovery of the soluble substance of pneumococcus is whether this substance is in any way responsible for the intoxication which attends pneumococcus infection. Studies to ascertain the answer to this question are being actively carried on at the present time but have not as yet progressed to the point at which a definite answer can be given. It may be said, however, that its toxicity is in no way comparable to that of diphtheria toxin. On the other hand, it possesses a degree of toxicity which, exhibited throughout the course of an infection, may account for the signs of intoxication in lobar pneumonia.

DISCUSSION.

The preceding experimental data have shown that a specifically reacting substance of pneumococcus origin occurs in the bacteria-free filtrates of young cultures of pneumococcus and also in the blood serum and urine of patients during lobar pneumonia. The occurrence of specifically precipitable substances in the cell-free filtrate of bacterial cultures has been known ever since the early obser-

vation of Kraus. It has been abundantly confirmed by other investigators with a variety of bacteria. In general, the presence of this precipitable substance has been demonstrated in culture fluids so old that an opportunity has occurred for cell death and disintegration and consequent solution of bacterial protein. In this paper it is shown that there is present in solution in the culture fluid in which pneumococcus is grown, a soluble substance in considerable amounts at a time when no cell death or disintegration has occurred. Consequently this substance does not represent dead dissolved bacterial protein, but the elaboration and passage into solution of a substance which is the product of the life activity of the cell. In addition to the evidence already cited in support of this fact, it has been demonstrated that the soluble substance is present in culture fluids in considerable concentration at a time when no hemolysin is present. This pneumococcus hemolysin is an intracellular body which does not appear in culture fluids until destruction of the bacterial cell has taken place; hence if the soluble substance described were purely of intracellular origin the curve of its concentration in culture fluids would be coincident with that of the hemolysin. This, however, is not the case, for the curve of hemolysin does not begin to rise until a time when the curve of the soluble substance has almost attained its maximum elevation.

The formation of a soluble substance by the pneumococcus on growth *in vitro* suggested the probability that an analogous substance would be formed on growth of the organism in the animal body and because of the readiness with which the substance passes into solution one would expect no difficulty in demonstrating it in the body fluids of experimentally infected animals. Examination of the blood and urine of rabbits infected with pneumococcus has shown this substance to be present in considerable quantities following intraperitoneal infection. Ascoli and Valenti[6] have demonstrated in the organisms of animals infected with anthrax a substance specifically precipitable with antianthrax serum. Bail[7] has shown the presence of a substance in the exudates of animals infected with anthrax which, when the fluids were freed from bacteria, increased the infectious

[6] Ascoli, A., and Valenti, E., *Centr. Bakteriol., Ite Abt., Ref.*, 1911, xlviii, 243.
[7] Bail, O., *Arch. Hyg.*, 1905, lii, 272; 1905, liii, 302.

power of anthrax bacilli. This substance he has called aggressin and he considers it to be an excretory product of the anthrax bacillus which favors the invasion of animal tissues by this organism. It is possible that the substances described by Ascoli and Valenti, and Bail are similar in the mechanism of their formation to the soluble substance produced by the pneumococcus. Although our study of other bacteria has been rather limited, it has been demonstrated that certain other species, such as meningococcus, *Bacillus typhosus*, and *Bacillus dysenteriæ*, also give rise to soluble material during their growth in fluid media.

A study of the serum of patients suffering from lobar pneumonia has shown that this soluble specific substance is also present in the circulating blood during the course of the disease in man. It gives a specific precipitin reaction with antipneumococcus serum corresponding in type to the organism with which the individual is infected. This soluble precipitable substance in human serum is less frequently present in demonstrable quantities than in the serum of experimentally infected animals. However, it has been found both when pneumococci are present in the circulating blood and when by blood culture organisms are absent. Complement fixation, as well as the precipitin reaction, may be used for the demonstration of this substance in serum. Although the soluble substance is relatively infrequently present in demonstrable quantities in the circulating blood, it is not unlikely, from the fact that the substance appears in a much larger percentage of cases in the urine, that it is much more commonly present in the blood than observed, but in quantities that are below the threshold of demonstration.

A study of the urine in 112 cases of lobar pneumonia and closely related respiratory diseases has shown that in 62.5 per cent of pneumonia due to Pneumococcus Types I, II, and III, a substance is excreted in the urine which reacts specifically with antipneumococcus serum of the type corresponding to the organism with which the individual is infected. This substance may appear as early as 12 hours after the initial chill, or may appear for the first time at a later stage of the disease, and may continue to be excreted for many days after recovery has occurred. In certain instances in which excretion occurred over a long time, its persistence in the urine could

be explained by delayed resolution, a condition which represents the passage of the acute pneumococcus infection of the lung into one of a more chronic character. In other instances of continued excretion not explainable on these grounds, the substance must have been stored in the tissues and must have passed into the circulating blood to be excreted by the kidneys without loss of its specific character. It is the rule to find the substance in the urine when pneumococcal septicemia exists. The amount of precipitable substance in the urine seems to be a measure of the severity of the infection. This fact may be dependent upon the quantity of the substance being directly proportional to the actual amount of infection or it may be that the amount of this substance formed bears some relationship to the virulence of the particular strain of pneumococcus responsible for the infection. Most of the instances which fail to show the presence of a precipitable substance in the urine recover, whereas the mortality is high among those in which its presence is demonstrable. If large amounts are excreted the outcome is usually fatal, unless this result is prevented by the administration of antipneumococcus serum. The specific precipitin test in the urine is therefore of considerable prognostic value. It may also be used in making a rapid diagnosis of the type of organism with which an individual is infected and in our experience a positive test in the urine is quite as reliable as the agglutination of the organism isolated from the sputum. The precipitin test in the urine, however, should not supplant the usual diagnostic technique in the determination of the type of pneumococcus.

11 years ago Fornet[8] claimed to have demonstrated in the serum and urine of patients suffering from typhoid fever a substance specifically precipitated by antityphoid serum. From what we now know it would seem likely that his observations were correct despite the fact that subsequent investigators failed to confirm them.

Ascoli[9] has shown that precipitinogen may pass the kidneys and appear in the urine where it exhibits its specific reaction. We have been able to show that if rabbits are inoculated intravenously with

[8] Fornet, O., *Münch. med. Woch.*, 1906, xxxviii, 1862.

[9] Ascoli, M., cited in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 750.

soluble pneumococcus material a specific precipitin reaction can be obtained in the urine within 24 hours and that the specific substance continues to be excreted for a number of days. In such an experiment the material injected contained no formed living pneumococci. Pettit[10] has demonstrated that if rats are injected with diphtheria toxin, this substance can be shown to be excreted in the urine in active form following the inoculation.

SUMMARY.

1. A specifically reacting substance of bacterial origin is present in the cell-free fluids of young cultures of pneumococcus. This substance is present when the organisms are growing at their maximum rate and undergoing little or no cell death, and consequently its presence is not dependent upon cell disintegration but represents the extrusion of bacterial substance by the living organism.

2. The blood and urine of rabbits experimentally infected with pneumococcus contain a similar specific soluble substance during the early hours of the infectious process.

3. Human beings suffering from lobar pneumonia have in their blood and more frequently in their urine a specific soluble substance of pneumococcus origin. The amount of this substance present in the urine varies in different individuals and the presence of a large amount is of unfavorable prognostic import. This specific precipitin reaction in the urine is of diagnostic value.

4. Rabbits injected with soluble pneumococcus material continue to excrete this substance for a considerable period of time.

5. The specifically soluble substance obtained from bacterial cultures and from the urine during infection is not destroyed by boiling, by precipitation with alcohol, acetone, or ether, or by trypsin digestion.

6. Studies are in progress at this time on the degree of toxicity and on the antigenic properties of the substance.

[10] Pettit, A., *Ann. Inst. Pasteur*, 1914, xxviii, 663.

ACIDOSIS AND ACID EXCRETION IN PNEUMONIA.*

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During the course of certain studies of the various factors of acid excretion,[1] 24 hour samples of urine from several normal and pathological subjects which were examined demonstrated that, in the absence of β-hydroxybutyric acid, the hydrogen ion concentration and titratable acidity are due largely to the ratio between acid and basic phosphates. Although in the main this hypothesis was quite well supported in the twenty-three samples analyzed, in one fatal case of pneumonia, over 60 per cent of the titratable acid could not be accounted for by the phosphates. This observation led to a more extended investigation of acidosis and acid excretion in acute lobar pneumonia, the results of which are presented in this paper.

There exists certain evidence which supports the idea that the metabolism during the febrile stage of pneumonia results in the production of considerable amounts of acid substances. Increased ammonia and titratable acid in the urine have been observed. Diminished carbon dioxide in the blood has been reported by several observers, notably by Peabody[2] who reviews the literature of the subject. Recently Lewis[3] has found the blood of patients with pneumonia to have a decreased affinity for oxygen, a characteristic of acidosis. Palmer and Henderson[4] noticed that during the fastigium of the disease larger amounts of sodium bicarbonate by mouth were necessary to reduce the acidity of the urine than is normally the case. Such facts as these, increased ammonia and acid excretion, low carbon dioxide in the blood, diminished affinity of the blood for oxygen, and retention of large amounts of alkali, indicate an excessive acid production during the febrile stage of the disease.

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* A brief report of this work was given before the American Society for Clinical Investigation, Atlantic City, N. J., May 7, 1917.

[1] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1914, xvii, 305; 1915, xxi, 37; *Arch. Int. Med.*, 1915, xvi, 109.

[2] Peabody, F. W., *J. Exp. Med.*, 1912, xvi, 701.

[3] Lewis, T., Lectures on the Heart, New York, 1915.

[4] Palmer and Henderson, *Arch. Int. Med.*, 1913, xii, 153.

Methods.

The several factors determined in the urine were the hydrogen ion concentration by the colorimetric method previously described,[5] titratable acidity by the method to be described presently, ammonia by Folin and Macallum's method,[6] and the total phosphates by the uranium acetate method. The combined carbon dioxide in the plasma was determined by the Van Slyke-Stillman-Cullen method.[7] As the method of titrating the urine has been modified for a special purpose and, so far as known, has not been used before, it will be described in detail. In the earlier part of the work the method as described by Henderson and Palmer[1] was used, but on account of the variation in the hydrogen ion concentration of the urine and because of the especial interest in the fluctuation in the difference between the total titratable acidity and the phosphate acidity of the urine, it seemed desirable to determine the titratable acidity between two fixed reactions, or in other words to estimate the quantity of acid or base involved in a specified change of hydrogen ion concentration at a level which is a function of the acids in question. By doing this it is possible to compare these differences at a fixed hydrogen ion concentration.

The hydrogen ion concentration[8] of 5.0 was selected as the acid endpoint because most acid urines in pneumonia, while not infrequently reaching this degree of acidity, seldom exceed it. The hydrogen ion concentration of 7.4 as the neutral point was for obvious reasons retained as in the titration previously described. Two titrations are necessary and are carried out as follows: 10 cc. of a 0.1 molal solution of monopotassium phosphate, 4 parts, and disodium phosphate, 25 parts

[5] Henderson and Palmer, *J. Biol. Chem.*, 1912–13, xiii, 393.

[6] Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.

[7] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 165.

[8] Throughout this paper the hydrogen ion concentration has been expressed by the negative exponent of the hydrogen ion concentration, that is, Sörensen's pH. The minus sign is omitted for convenience; for example, a hydrogen ion concentration of 5 means a pH of 5 or an H^+ of 10^{-5}. It should be remembered that an actual increase in hydrogen ion concentration is indicated by a decrease in this exponential figure.

(yielding a hydrogen ion concentration of 7.4) are introduced into a 250 cc. flask, diluted to 250 cc. with distilled water, and 0.2[9] cc. of a 1 per cent aqueous solution of neutral red is added. 10 cc. of urine are similarly diluted, neutral red is added, and 0.1 N sodium hydrate is run in until the color matches that of the phosphate solution. The second part of the titration is accomplished by introducing 10 cc. of a 0.14 molal solution of acetic acid, 23 parts, and sodium acetate, 46 parts (yielding a hydrogen ion concentration of 5.0) into a 250 cc. flask, diluted to 250 cc. with distilled water, and five drops of a 1 per cent aqueous solution of sodium alizarin sulfonate are added. 10 cc. of urine are similarly diluted, sodium alizarin sulfonate is added, and 0.1 N hydrochloric acid run in until the color matches that of the acetic acid-sodium acetate solution. In matching the color of the alizarin solutions, especially when the urine is highly colored, it has been found desirable to place a flask containing 10 cc. of urine diluted as for titration but without indicator, behind the flask containing the standard acetic acid-sodium acetate solution with indicator. Also a flask with distilled water is similarly placed back of the urinary sample which is being titrated. The titration must be carried out in front of a window with good light. The titratable acidity in 0.1 N cc. between the hydrogen ion concentration of 5.0 and the hydrogen ion concentration of 7.4 is the sum of these two titrations.

With the above data available, it is possible to compare the actual titratable acidity with that which the phosphates alone would give. The calculation of the part of the phosphates is simple. In the equilibrium[10] $H^+ = K \times \frac{HA}{BA}$ approximately, where H^+ is the hydrogen ion concentration, K the ionization constant k for the acid divided by α, the degree of ionization of the salt of the acid, HA the undissociated acid, and BA the salt of the acid, H^+ is determined and K is known, hence $\frac{HA}{BA}$ may be easily calculated; and if the total phos-

[9] It has been found convenient and satisfactory to use a dropping bottle for adding the indicator. Care should always be taken to add the same size and number of drops to standard and urine samples. Three to five drops are sufficient.

[10] Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 427.

phates are known the amount of HA and BA can be estimated. Reference to Sörensen's measurements shows that this titration is sufficiently accurate within the ranges of hydrogen ion concentration involved in the present investigation. It is appreciated, however, that the presence of electrolytes and variation in the concentration of the urine combine to decrease the accuracy of the titration but not to a degree sufficient to alter the conclusions reached. An example of the calculation follows.

The pH = 5.3 or $H^+ = 50 \times 10^{-7}$, titratable acidity amounts to 200 cc. 0.1 N acid, total phosphates in terms of phosphorus pentoxide is 1.0 gm. When the acid is monobasic phosphate $K = 2.5 \times 10^{-7}$. We have then, from the above equilibrium, $50 \times 10^{-7} = 2.5 \times 10^{-7} \times \frac{HA}{BA}$, whence $\frac{HA}{BA} = \frac{20}{1}$; that is, 96 per cent of the phosphate exists as the monobasic phosphate. 1 gm. of phosphorus pentoxide corresponds to 141 cc. of 0.1 N monobasic phosphate. Hence in a mixture of mono- and dibasic phosphates containing 1.0 gm. of phosphorus pentoxide and having a hydrogen ion concentration of 5.3, 135 cc. exist as the acid and 6 cc. in the basic form. As our method of titrating the urine carries the reaction to a hydrogen ion concentration of 7.4 or when the ratio $\frac{MH_2PO_4}{M_2HPO_4} = \frac{4}{25}$, 14 per cent of the phosphate is in the acid form, then the per cent of the acid phosphate which actually takes part in the titration from a hydrogen ion concentration of 5.3 to a hydrogen ion concentration of 7.4 is 96 − 14 or 82 per cent. Hence 82 per cent of 141 cc. amounts to 115 cc. and the difference between the actual acidity and acidity calculated from the phosphates is 200 − 115 or 85 cc.

This difference is probably due largely to free organic acids and will be so designated in the discussion following. In Table I the percentage of acid phosphate for any hydrogen ion concentration between 5.0 and 7.4 has been computed, as well as the amounts of acid phosphate entering into the titration between any hydrogen ion concentration within these limits and 7.4.

TABLE I.

Ratio of Acid, HA, to Basic, BA, Phosphate at a Hydrogen Ion Concentration Varying between 7.4 and 4.9 with the Percentage of Acid Phosphate Taking Part in Titration When Carried to a Hydrogen Ion Concentration of 7.4. $K = 2.5 \times 10^{-7}$. $pH = 7.4$ Which Is the Reaction of Normal Blood.

pH.	$H^+ \times 10^{-7}$.	$\frac{HA}{BA}$	HA.	HA–14.
			per cent	*per cent*
7.4	0.4	$\frac{4}{25}$	14	0
7.2	0.6	$\frac{6}{25}$	19	5
7.0	1.0	$\frac{10}{25}$	29	15
6.9	1.3	$\frac{13}{25}$	34	20
6.8	1.6	$\frac{16}{25}$	39	25
6.7	2.0	$\frac{20}{25}$	45	31
6.6	2.5	$\frac{25}{25}$	50	36
6.5	3.2	$\frac{32}{25}$	56	42
6.4	4.0	$\frac{40}{25}$	62	48
6.3	5.0	$\frac{50}{25}$	67	53
6.2	6.3	$\frac{63}{25}$	72	58
6.1	8.0	$\frac{80}{25}$	76	62
6.0	10.0	$\frac{100}{25}$	80	66
5.9	13.0	$\frac{130}{25}$	84	70
5.8	16.0	$\frac{160}{25}$	87	73
5.7	20.0	$\frac{200}{25}$	89	75
5.6	25.0	$\frac{250}{25}$	91	77

TABLE I—*Concluded.*

pH.	$H^+ \times 10^{-7}$.	$\frac{HA}{BA}$	HA.	HA–14.
			per cent	*per cent*
5.5	32.0	$\frac{320}{25}$	93	79
5.4	40.0	$\frac{400}{25}$	94	80
5.3	50.0	$\frac{500}{25}$	95	81
5.2	63.0	$\frac{630}{25}$	96	82
5.1	80.0	$\frac{800}{25}$	97	83
5.0	100.0	$\frac{1000}{25}$	98	84
4.9	120.0	$\frac{1200}{25}$	98	84

OBSERVATIONS.

For a preliminary survey 24 hour samples of fresh urine from six normal individuals and seventeen cases representing a wide variety of pathological conditions selected from the medical wards of the Massachusetts General Hospital were examined, the results of which appear in Table II.

Case 15 stands out very prominently with 323 cc. of 0.1 N free organic acid, while of the remaining cases 10 showed less than 50 cc., 7 less than 100 cc., 4 less than 150 cc., and 1 normal individual showed as much as 187 cc. It is possible that the high value in Case 6 may be explained partly by the high hydrogen ion concentration and partly by the size of the individual, or, indeed, by a possible abnormality in metabolism. Cases 16 and 23 had high temperatures but showed only small amounts of free organic acid when compared with the total acidity. The ammonia excretion in Case 15 is the highest in the series but is not excessive.

TABLE II.

Normal and Pathological Individuals.

No.	Volume of urine.	Weight.	pH.	Acid 0.1 N.	Ammonia 0.1 N.	Acid + ammonia 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Free organic acid at the determined pH,* 0.1 N.	Remarks.
	cc.	*kg.*		*cc.*	*cc.*	*cc.*	*gm.*	*cc.*	*cc.*	
1	1,420	94	6.0	336	450	786	2.45	228	108	Normal.
2	1,560	64	6.1	272	375	647	2.31	202	70	"
3	2,000	79	6.3	320	590	910	3.95	295	25	"
4	2,100	89	6.7	143	458	601	2.33	102	41	"
5	1,100	73	5.7	386	418	804	3.05	323	63	"
6	1,000	91	5.4	415	350	765	2.02	228	187	"
7	2,000	61	5.9	186	280	466	1.34	132	54	Chronic alcoholism.
8	740	80	6.1	126	420	546	1.55	135	−9	Arteriosclerosis.
9	1,700	68	5.7	180	293	473	1.40	148	32	Pernicious anemia.
10	2,650	69	7.0	64	382	446	2.16	46	18	Cardiorenal disease.
11	750	78	5.3	125	252	377	0.63	72	53	" "
12	1,290	44	6.0	184	160	344	1.45	135	49	Pleurisy with effusion.
13	2,440	50	6.3	176	346	522	2.02	151	25	" " "
14	2,500	51	6.0	182	390	572	1.58	147	35	Unexplained edema of lower legs.
15	2,360	70	5.3	580	750	1,330	2.25	257	323	Acute lobar pneumonia.
16	580	62	5.3	242	550	792	0.90	103	139	" endocarditis. Temperature 103°F.
17	1,540	68	5.5	510	595	1,105	3.38	377	133	Diffuse sclerosis of central nervous system.
18	1,120	66	6.3	210	392	602	1.95	146	64	Cholelithiasis.
19	1,350	74	6.2	155	141	296	1.18	97	58	Convalescent; typhoid fever.
20	560	63	5.6	145	170	315	0.88	96	49	Cholelithiasis.
21	2,500	50	6.7	100	380	480	1.20	52	48	Chronic constipation.
22	900	57	5.7	206	355	561	1.10	116	90	Diabetes (ferric chloride reaction negative).
23	1,775	60	5.8	445	683	1,128	3.24	334	111	Typhoid fever. Temperature 104.0°F.

* All samples gave no color with ferric chloride.

Normal Individuals.—Four normal subjects of widely varying weights were examined. Very little difference between the individuals was found, hence only one of the protocols is given (Table III).

TABLE III.

Factors Determined on a Normal Individual, Weight 70 Kilos, for 9 Consecutive Days.

Volume of urine.	pH.	Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.
cc.		*cc.*	*cc.*	*gm.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
1,080	5.5	318	378	1.63	182	190	136	188	346
1,270	5.6	324	414	1.81	196	212	128	202	432
1,000	5.6	320	400	1.86	202	220	118	180	280
1,090	5.5	175	251	1.70	189	202	−14	49	344
600	5.5	180	246	1.00	112	118	68	128	132
1,070	6.6	257	460	1.92	96	228	161	232	293
1,930	6.4	250	425	1.52	103	180	147	245	367
1,015	6.0	142	234	0.85	79	100	63	134	238
1,510	5.7	288	378	1.72	180	202	108	176	417

There is considerable variation in the amounts of free organic acid both when calculated from the hydrogen ion concentration at which the urine was passed and when estimated at the hydrogen ion concentration of 5.0. The hydrogen ion concentration of the case selected varies very little although the free organic acid varies considerably. In general, as the hydrogen ion concentration increases in value, that is, as the urine becomes more alkaline, the amount of organic acid present in the free state is less. On the other hand, as the reaction approaches a hydrogen ion concentration of 5.0 the organic acid fraction increases very rapidly.

Individuals with Acute Lobar Pneumonia.—In all, thirty cases of acute lobar pneumonia, involving the analysis of 325 24 hour samples of urine, were studied. It is not necessary to report in detail all of the protocols of the series. Certain cases have been selected as representative of the various conditions found in the group (Tables IV, V, VI, VII, and VIII). In general it may be said that the more severe the intoxication the greater the amounts of free organic acid at the hydrogen ion concentration of 5.0 which are present. The type of organism[11] was determined in twenty-three of the cases, showing 4

[11] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

with Type I, 11 with Type II, 4 with Type III, and 4 with Type IV.

One of the four with Type I infection showed an increase in the free organic acid but in small amount only. In six of the Type II group there was a very marked increase, in three a moderate increase of the free organic acid, while two were without any significant change. There was no increase in the acid excretion in any of the Type III cases. This finding is not what might be expected *a priori* because this type of infection has proved to have the highest mortality of all, 50 per cent of the cases being fatal. One of the group studied died, the protocol of whom is given in Table V. Two of the Type IV cases showed some increase in the free organic acid excretion; the others did not.

The case reported in Table IV was chosen as an example of those individuals who showed no particular signs of either acidosis or increase in acid excretion from the laboratory or clinical standpoint. The total amount of acid excreted is not excessive; although the ammonia excretion on the 6th and 7th days of the disease just before the crisis is greater than on the days following, it is not large. At no time is the combined carbon dioxide of the plasma below normal. The lower limit of normal given by Van Slyke, Stillman, and Cullen is 55 volume per cent, which corresponds to an alveolar air of about 38 mm. carbon dioxide tension. The acidity of the urine fell at the time of the crisis, and this occurred in most of the cases having a definite crisis.

The protocol of a fatal case appearing in Table V is given to illustrate the unexpected finding in the Type III infection. Although the intoxication in this infection was very great, there was no marked increase in the free organic acid nor was there any other evidence of acid intoxication. The ammonia and acid excretion, as well as the combined carbon dioxide in the plasma, were well within normal limits.

In Table VI are given the data of a case with Type II infection which is a fair example of what frequently occurs in the more severe infections.

At the time of the crisis the amounts of acid not accounted for by the phosphates fall off and quite regularly during convalescence

TABLE IV.

Hospital No. 2,852; Male; Age 33 Years. Process Confined to the Left Lower Lobe. Type I Infection. Blood Culture Positive. Treated with Serum. A Severe Chill Followed the Fifth Treatment, after Which the Temperature Fell by Crisis.

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a Acid 0.1 N.	b Acid pH 5.0-7.4, 0.1 N.	c Phosphorus pentoxide.	d Phosphorus pentoxide acid 0.1 N, pH 7.4.	e Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	f (a-d) Free organic acid at the determined pH, 0.1 N.	g (b-e) Free organic acid at the pH of 5.0, 0.1 N.	h Ammonia 0.1 N.	i Combined carbon dioxide in plasma. Volume per cent.	Remarks.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.		
5	104.5	124	54												
	102.5	108	46	1,060	5.4	460	533	2.50	282	296	178	237	650	58.6	175 cc. of antipneumococcus serum.
6	103.6	106	48												
	100.8	96	40	1,105	5.5	196	265	0.51	57	60	139	205	910	60.6	300 cc. of antipneumococcus serum. Chill.
7	106.5	154	46												
	98.2	72	30	1,185	5.7	250	440	1.20	127	142	123	298	975		
8	100.5	88	40												
	99.5	72	28	985	6.1	145	334	0.74	65	88	80	246	630	61.7	
9	99.5	76	40												
	99.2	60	24	865	6.6	152	372	1.10	43	130	109	242	430		
10	100.4	76	36												
	98.5	60	28	1,200	6.4	370	665	3.00	204	356	166	309	605		
11	99.5	78	28												
	98.5	64	20	900	5.8	310	436	1.80	184	212	126	224	790	62.2	
12	100.4	80	32												
	99.0	74	28	900	5.6	225	336	1.27	138	150	87	186	378		
13	100.4	88	28												
	99.4	80	20	625	5.6	73	145	0.83	90	98	−17	47	295	69.1	
14	101.6	100	40												
	100.0	80	20	1,230	5.6	325	450	1.68	182	198	143	252	410		Beginning of serum disease.
15*	102.6	100	36												
	100.5	80	20	850	5.5	278	356	1.32	147	156	131	200	283		

* This case was followed for a week longer but as there was nothing of note in the data, they are not given.

TABLE V.

Hospital No. 2,593; Male; Age 37 Years. Process at the Entrance of the Right Lower Lobe Extending 2 Days before Death to the Entire Right Lung. Type III Infection. Blood Culture Positive. Treated with Optochin. Died on the 9th Day of the Disease.

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a Acid 0.1 N.	b Acid pH 5.0–7.4, 0.1 N.	c Phosphorus pentoxide.	d Phosphorus pentoxide acid 0.1 N, pH 7.4.	n Phosphorus pentoxide acid 0.1 N, pH 5.0–7.4.	f (a–d) Free organic acid at the determined pH, 0.1 N.	g (b–e) Free organic acid at the pH of 5.0, 0.1 N.	h Ammonia 0.1 N.	i Combined carbon dioxide in plasma. Volume per cent.	Remarks.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.		
3	104.0	112	38												
	102.5	102	32	1,425	5.8	184	395	1.57	162	186	22	209	427	54.1	
4	104.0	116	42												
	103.0	100	36	1,675	5.7	218	400	1.71	180	202	38	198	524	59.8	
5	103.0	112	44												
	102.0	100	32	2,200	5.6	396	600	3.34	363	396	33	204	880		
6	103.0	112	40												
	102.0	104	32	2,040	5.2	388	470	3.04	352	360	36	110	885	51.3	
7	102.0	120	48												
	102.0	102	32	1,510	5.2	348	410	2.60	300	308	48	102	800	50.4	Died 2 days later.

keep within normal limits. Early in the investigation before the free organic acid fraction was compared at the fixed point of pH = 5.0, this remarkable decrease was somewhat misleading due to the fact that the hydrogen ion concentration of the urine changed to the nearly neutral point at the crisis. On the 7th day of the disease the free organic acid excreted in 24 hours amounted to 760 cc., while in the days following the limits were between 220 and 351 cc. There was also a marked increase in the ammonia excretion, amounting to 2,050 cc. on the 7th day, rapidly coming down to normal values

TABLE VI.

Hospital No. 2,865; Male; Age 23 Years. Process Involving the Entire Left Lung. Type II Infection. Blood Culture Negative. Treated with Optochin. Recovery.

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a–d)	g (b–e)	h	i
						Acid 0.1 N.	Acid pH 5.0–7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0–7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	
4	106.0	120	48											
	105.4	110	32	1,740	5.2	588	640	1.47	170	174	418	466	432	62.5
5	104.8	116	48											
	103.4	104	40	2,350	5.0	910	910	4.00	474	474	436	436	595	61.4
6	103.0	100	44											
	101.0	84	32	3,510	5.8	990	1,380	7.00	723	828	267	552	1,580	65.3
7	100.5	102	48											
	99.2	72	30	2,300	6.2	660	1,120	3.04	248	360	412	760	2,050	66.8
8	101.2	76	40											
	100.0	70	28	1,555	6.5	202	507	1.32	78	156	124	351	1,130	60.8
10	101.2	80	30											
	99.8	46	24	925	5.8	390	565	2.42	250	287	140	278	694	63.8
11	101.0	76	32											
	98.0	60	24	650	5.6	387	500	2.36	256	280	131	220	700	
12	99.5	68	24											
	99.0	58	20	900	5.8	400	532	2.63	271	312	129	220	510	
13	99.8	72	24											
	98.3	60	20	925	5.5	500	563	2.75	306	325	194	238	495	
14	99.2	64	22											
	98.6	60	20	1,688	5.4	450	515	2.10	236	248	214	267	580	
15*	99.5	96	22											
	99.0	60	20	1,200	5.6	412	460	2.03	220	240	192	220	327	

* This case was followed a week longer but as the data contained nothing of especial interest they are not given.

after the crisis. A decided fall in urinary acidity occurred after the crisis. Throughout the entire course of the disease, however, the combined carbon dioxide in the plasma remained normal, indicating the ability of the organism to cope with the increased acid production.

The case reported in Table VII excreted the largest amount of free organic acid in 24 hours of any of the cases observed. On the 4th day of the disease 1,165 cc. of the total acidity of 1,800 cc. at a hydrogen ion concentration of 5.0 were unaccounted for by the phosphates. The intoxication was intense. That the mechanism for regulating the acid-base equilibrium was sufficient is proved by the combined carbon dioxide in the plasma. Only 2 hours before death it was 62.5 volume per cent, the lowest at any time during the disease.

TABLE VII.

Hospital No. 2,869; Male; Age 31 Years. Process in the Right Lower Lobe. Type II Infection. Blood Culture Positive. Treated with Serum and Optochin. Died.

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a–d)	g (b–e)	h	i
						Acid 0.1 N.	Acid pH 5.0–7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0–7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	
3*	103.4	120	48											
	103.0	108	32	4,340	5.5	1,600	1,900	9.65	1,070	1,140	530	760	737	64.1
4*	103.3	124	48											
	102.4	108	30	3,160	5.6	1,380	1,800	5.35	580	635	800	1,165	1,730	64.1
5*†	104.0	128	52											
	102.0	114	30	1,900	5.9	475	820	2.88	284	340	191	480	688	63.5

* 190 cc. of antipneumococcus serum.

† Died on the 6th day of the disease. The combined carbon dioxide in the plasma 2 hours before death was 62.5 volume per cent.

TABLE VIII.

Hospital No. 2,634; Male; Age 42 Years. Process Confined to the Right Lower Lobe. Type IV Infection. Blood Culture Negative. Treated with Optochin. Recovery.

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
						Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	
7	104.8	106	44											
	103.4	90	32	1,975	5.3	623	730	4.10	470	486	153	244	1,040	60.7
8	104.6	96	48											
	103.0	82	30	1,580	5.4	350	440	2.24	252	266	98	174	1,060	60.7
9	104.0	84	46											
	102.5	82	32	1,775	5.2	370	497	2.62	304	310	66	187	1,200	52.8
10	103.5	88	40											
	100.0	72	32	2,560	5.7	294	460	2.62	278	310	16	150	1,800	64.5
11	102.5	70	30											
	99.0	56	24	1,550	5.7	268	392	1.83	193	216	75	176	1,180	68.3
12	99.2	72	32											
	98.6	58	24	1,415	5.5	318	410	2.20	246	261	72	149	900	
13	99.2	76	32											
	98.6	56	24	1,630	5.6	394	542	3.02	328	358	66	184	625	
14	99.6	84	26											
	98.6	58	18	1,665	5.5	372	478	2.45	273	290	99	188	500	
15	99.4	80	20											
	98.6	64	18	1,715	5.2	394	437	2.50	290	296	104	141	620	54.1
17	99.0	70	24											
	98.6	52	18	1,490	5.0	540	540	3.12	370	370	170	170	690	
18	99.6	80	18											
	98.8	58	18	1,300	5.0	430	430	2.50	296	296	134	134	533	

TABLE VIII—*Concluded.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a–d)	g (b–e)	h	i
						Acid 0.1 N.	Acid pH 5.0–7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0–7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	
20	99.6	80	20											
	98.7	60	18	1,500	5.0	480	480	2.74	325	325	155	155	540	
21	99.6	80	20											
	98.8	56	18	1,380	5.0	400	400	2.60	308	308	92	92	458	
23	100.0	80	20											
	98.8	64	20	1,080	5.0	260	260	1.67	198	198	62	62	290	49
24	100.0	80	24											
	99.2	56	18	1,415	5.0	396	396	2.28	270	270	126	126	315	
25	100.2	72	18											
	99.2	60	18	1,140	5.0	348	348	2.32	280	280	68	68	413	
27	99.5	84	18											
	99.2	70	18	1,135	5.0	330	330	1.83	217	217	113	113	330	

The individual with a Type IV infection reported in Table VIII revealed a condition not found in any of the other cases studied. While there were only small amounts of free organic acid excreted during the fastigium of the disease and in convalescence, the ammonia excretion was very high until after the crisis, when the 24 hour values became normal. There was never any significant lowering of the combined carbon dioxide in the plasma.

DISCUSSION.

The facts brought out by the investigation are that in many, usually the more severe, cases of acute lobar pneumonia there are excreted during the fastigium of the disease considerable quantities of an organic acid which is free at a hydrogen ion concentration of 5.0, and that

there is seldom a severe grade of acidosis as estimated by the amount of combined carbon dioxide in the plasma. The nature and biological importance of this organic acid are not without interest. Certain possibilities are suggested. Because of its prevalence in many conditions where there is abnormal metabolism, β-hydroxybutyric acid was searched for, although all specimens examined had a negative or at most a very faint ferric chloride reaction. The ionization constant of β-hydroxybutyric acid is 2×10^{-5}, therefore one-third of the acid is free in a urine with a hydrogen ion concentration of 5.0. Quantitative estimates revealed insignificant amounts. This is not surprising when one considers, for instance, that in Table VI on the 7th day of the disease the acid unaccounted for amounts to 760 cc. and if it were all due to β-hydroxybutyric acid there would be present a total of 24 gm. of the acid, an amount which is seldom encountered except in the more severe grades of acidosis in diabetes. Hippuric acid with an ionization constant of 2.2×10^{-4}, acetoacetic acid with an ionization constant of 1.5×10^{-4}, and lactic acid with an ionization constant of 1.4×10^{-4} whereby only 5 to 7 per cent of an acid can exist free at a hydrogen ion concentration of 5.0 could hardly be expected to account for any considerable quantities of free organic acid. Acetic acid, the ionization constant of which is 1.9×10^{-5}, exists about one-third free at a hydrogen ion concentration of 5.0, hence it becomes a possibility. While uric acid with an ionization constant of 1.5×10^{-6} may be 87 per cent free at a hydrogen ion concentration of 5.0, the total amount of this substance is easily estimated and has never been found to account for more than a few cubic centimeters of the free acid. Nor can the conjugated sulfuric acid be responsible for any large quantities as shown by several ethereal sulfate determinations. The oxy- and other less well known acids are possibilities, but as their ionization constants are not known nothing definite can be stated about them. In Table VIII the acid is apparently a fairly strong one as shown by the high ammonia without much free acid or high total phosphates.

As it has been shown that normally at a hydrogen ion concentration of 5.0 there exists a certain titratable acidity that cannot be accounted for by the phosphates, it is possible that the increase in this value during pneumonia may be due simply to an increase of a normal

constituent of the urine. On the other hand, the possibility of abnormal oxidation products leads one to suspect that there may exist some pathological substances to account for the phenomenon and this in part has been borne out by our investigation. If this is the case, its part, if any, in the intoxication encountered in the disease is of much interest. Considerable investigation of the nature and significance of the increase in the free organic acid production of the urine during pneumonia has been carried out, and will form the subject of a future communication.

SUMMARY.

There is excreted in the urine of subjects ill with acute lobar pneumonia a large amount of organic acid which is free at a hydrogen ion concentration of 5.0.

Acidosis as determined by the combined carbon dioxide in the plasma is seldom, if ever, severe.

FURTHER STUDIES ON THE EPIDEMIOLOGY OF LOBAR PNEUMONIA.

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Previous studies on the epidemiology of lobar pneumonia have shown that although pneumococcus is present in the mouths of about 50 per cent of normal individuals, it is extremely rare to find pneumococcus of Type I or Type II in the normal mouth except in individuals who have been in intimate association with patients suffering from lobar pneumonia due to organisms of these types. The observations of Dochez and Avery[1] suggest that in many instances lobar pneumonia may be due to contact infection. The source of such infection may be either an individual who has recovered from lobar pneumonia but who still harbors in his mouth the infectious microorganism, or a healthy carrier who has acquired the organism from association with a case of lobar pneumonia.

In the present paper are reported, first, the varieties of pneumococcus isolated from cases of lobar pneumonia admitted to the Hospital of The Rockefeller Institute during the past 5 years, second, the varieties of pneumococcus found in the mouths of normal individuals, third, a study of the types of pneumococcus present in the mouth secretions of members of households where cases of Type I and Type II pneumonia have occurred, fourth, the types of pneumococcus obtained from the dust of rooms in which no case of pneumonia is known to have occurred, fifth, the types of pneumococcus obtained from the dust in homes where pneumonia of Type I or Type II has occurred, and sixth, two epidemics of pneumonia.

[1] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxii, 105.

Varieties of Pneumococcus Concerned in the Production of Lobar Pneumonia.

During the past 5 years a study has been carried on of the types of pneumococcus isolated from 454 cases of lobar pneumonia admitted to the wards of the Hospital of The Rockefeller Institute. These organisms have been isolated from sputum, from blood cultures, or directly from the lung. In many instances they have been isolated from more than one of these sources and classified in the biological group already described by Dochez and Gillespie.[2] The results of this classification are shown in Table I.

TABLE I.

Types of Pneumococcus Causing Lobar Pneumonia in 454 Cases.

Pneumococcus.	Incidence.	
		per cent
Type I	151	33.26
" II	133	29.29
" II a	6	1.32
" II b	4	0.88
" II x	9	1.98
" III	59	12.99
" IV	92	20.26

The incidence of Types II a, II b, and II x, subvarieties of Type II, described by Avery,[3] has only been determined during the last 2 years.

Varieties of Pneumococcus in the Normal Mouth.

Pneumococci were isolated in 116 instances from the saliva of 297 normal individuals in whom no history of contact with a previous case of lobar pneumonia could be obtained. In five instances two types of pneumococci were isolated from the same individual. Pneumococcus Type I was isolated only once; Pneumococcus Type II was not found among these 297 healthy individuals. Among the 39 per cent showing pneumococcus in the mouth secretions, the dis-

[2] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.
[3] Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

TABLE II.

Types of Pneumococcus Isolated from the Saliva of Normal Individuals Not in Direct Contact with Lobar Pneumonia.

Pneumococcus.	Incidence.	
		per cent
Type I	1	0.8
" II	0	0
" II a	1	0.8
" II b	7	5.8
" II x	14	11.6
" III	34	28.1
" IV	64	52.9
Pneumococcus present	116	
" absent	181	
	297	

tribution of types is in striking contrast to that which is found among individuals in direct contact with disease. In Table IV are given the results of the examinations of 184 normal individuals known to be in contact with a case of lobar pneumonia due to pneumococci of Types I and II. Among 107 normal individuals known to be intimately associated with a Type I infection, 15 per cent showed pneumococcus of the same type as that causing disease. Of the remaining 77 individuals, all of whom were in direct contact with a Type II infection, this organism was isolated in 5 instances (6 per cent). In all, pneumococci of the more strictly disease-producing types, I and II, were found in 11 per cent of individuals in direct contact with cases of lobar pneumonia due to these types; while among 297 individuals in whom no history of previous contact could be obtained, pneumococci of Types I and II were found in only 0.8 per cent.

The pneumococcus is found in the mouths of many normal individuals. In some the same type of pneumococcus is present constantly, while in others the types may vary from time to time or entirely disappear. In Table III is presented a study of the types of pneumococcus recovered from the saliva of 942 healthy persons.

From the saliva of 942 normal individuals including those in contact with lobar pneumonia one or more types of pneumococcus were

TABLE III.

Types of Pneumococcus Isolated from the Sputum of Normal Individuals, Including Those in Direct Contact with Lobar Pneumonia.

Pneumococcus.	Incidence.	
		per cent
Type I	34	7.0
" II	22	4.5
" II a	1	0.2
" II b	26	5.3
" II x	47	9.7
" III	85	17.5
" IV	271	55.8
Pneumococcus present	450	
" absent	492	
	942	

isolated in 450 instances, or 47 per cent. In 492 individuals no pneumococci could be recovered at the time of examination. Type I pneumococcus was isolated from 34 individuals. 33 of these persons had recently been in close association with a case of lobar pneumonia due to the same type. In only one instance was there no history of contact. From 22 individuals Type II pneumococcus was obtained. 19 of these persons had recently been intimately associated with a case of lobar pneumonia of Type II. It is significant that only once was pneumococcus of Type II a isolated from a normal mouth. Yet this organism was responsible for six cases of pneumonia, or 1.32 per cent. On the other hand, Pneumococcus Type II b was found 26 times and Pneumococcus Type II x 47 times in normal mouths. In view of the relative frequency of Types II b and II x in normal mouths, these organisms, as those of Types III and IV, must be considered as normal inhabitants of the healthy mouth. It is not infrequent to find two types of pneumococcus at the same time in one mouth. But as yet a Type I and a Type II pneumococcus have never been found simultaneously in the same mouth.

Incidence of the Carrier Condition in Normal Individuals in Contact with Patients Suffering from Lobar Pneumonia.

Dochez and Avery[1] have shown that many individuals who come in contact with active cases of lobar pneumonia due to infection with Pneumococcus Type I and Type II carry in their mouth secretions a pneumococcus of the same type as that causing the disease with which they have been associated. In order to determine the frequency with which normal individuals acquire these types of pneumococcus, the following study was carried on.

A nurse was sent to the home as soon as possible after a patient suffering from pneumonia due to Type I or Type II had been admitted to the hospital. Specimens of sputum were collected from all members of the household; *i.e.*, all persons who had come in contact with the patient. In Table IV are presented the results of this study. This table includes all the cases which were studied from this point of view. It shows the incidence and type of pneumococcus found among the members of 52 households. From 28 of these homes came patients with lobar pneumonia due to Type I infection. Among the 107 members of these families 16, or 15 per cent, showed Type I pneumococcus. One or more positive contacts were found in 10 (36 per cent) of the homes. Type II pneumococcus was isolated from only 1 individual. 77 individuals from 24 homes in which a case of Type II pneumonia had occurred were studied. From 5, or 6 per cent, a Type II pneumococcus was isolated. In 5, or 21 per cent, of the 24 homes a positive contact was found. A Type I pneumococcus was not found among these individuals. Altogether, from a total of 52 cases of lobar pneumonia due to Type I and Type II, 15, or 28 per cent, gave rise to the carrier condition in at least one of their immediate associates. The total number of households examined was 52, and in 15, or 28 per cent, there were one or more persons who showed pneumococcus of Type I or Type II in their sputum. Of the 184 persons who composed these households 21, or 11 per cent, showed either a Type I or a Type II pneumococcus.

The average carrying period for Type I was 25 days, and for Type II 43 days. The carrying period was roughly estimated by taking the middle date between the last positive sputum and the first negative one. If the contact was still positive at the last examination, a plus sign is added.

TABLE IV.

Incidence of the Carrier Condition in Healthy Individuals in Contact with Lobar Pneumonia.

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				days
2,746	I	Father.	IV	
		Mother.	No pneumococcus.	
		Sister T.	IV	
		" A.	IV	
2,751	II	Wife.	II x and III	
		Sister.	No pneumococcus.	
		Brother.	IV	
		Brother-in-law.	II b and IV	
2,786	II	Husband.	II x	
		Son.	IV	
		Daughter.	IV	
		Friend.	II	48
2,804	I	" Mr. O.	IV	
		" Mrs. O.	IV	
		" G.	II x	
		" H.	IV	
2,814	I	Wife.	IV	
		Brother.	II b	
2,815	I	Husband.	No pneumococcus.	
		Daughter R.	I	6
		" H.	No pneumococcus.	
		Friend.	II x	
2,816	I	Wife.	No pneumococcus.	31
		Son.	I	
2,821	I	Sister-in-law.	No pneumococcus.	
		Brother.	II	Undetermined.
		Friend.	II b	
2,824	I	Sister.	No pneumococcus.	
2,825	II	Wife.	II	48
		Son-in-law.	No pneumococcus.	
		Daughter.	II x	
2,827	II	Wife.	No pneumococcus.	
		Friend.	II b and III	
2,834	II	Sister.	IV	
		Niece T.	IV	
		" M.	IV	
		Nephew H.	II b and IV	
		Friend C.	II b " III	
		" D.	II b " IV	

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				days
2,852	I	Mother.	No pneumococcus.	
		Sister M.	IV	
		" A.	No pneumoccocus.	
		Brother L.	" "	
		" I.	II x	
2,853	II	Mother.	No pneumococcus.	
		Father.	IV	
		Sister E.	No pneumococcus.	
		" A.	" "	
		Brother.	" "	
2,854	II	Wife.	" "	
		Daughter H.	" "	
		" M.	" "	
		" I.	III and IV	
		Friend.	II	Undetermined.
2,858	I	Wife.	No pneumococcus.	
		Daughter.	IV	
		Son R.	II x and IV	
		" I.	IV	
2,868	II	Mother.	II b	
		Brother E.	No pneumococcus.	
		" A.	" "	
		" W.	" "	
2,869	II	Wife.	IV	
		Daughter A.	No pneumococcus.	
		" I.	" "	
		Brother.	" "	
2,874	I	Friend F.	II x	
		" J.	III	
		" H.	II x	
2,879	II	Wife.	No pneumococcus.	
2,880	I	Mother.	IV	
		Father.	No pneumococcus.	
		Brother W.	IV	
		" R.	III	
		Sister.	I and IV	41
2,881	II	"	IV	
		Brother.	No pneumococcus.	
		Brother-in-law.	II x and III	
		Niece.	IV	

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				days
2,883	I	Son.	No pneumococcus.	
		Daughter.	" "	
		Friend S.	" "	
		" Y.	II x	
2,885	II	Maid J.	IV	
		" A.	No pneumococcus.	
		" M.	" "	
2,886	II	Sister C.	" "	
		" E.	II x	
		Niece.	No pneumococcus.	
		Brother-in-law.	IV	
2,890	II	Wife.	No pneumococcus.	
2,891	I	Sister.	" "	
		Niece E.	" "	
		" M.	" "	
		Brother-in-law.	" "	
2,892	II	Wife.	" "	
		Daughter X.	II x	
		" M.	III	
		Son.	II x	
		Boarder.	No pneumococcus.	
2,896	II	Mother.	IV	
		Sister.	IV	
		Brother.	II x	
2,901	I	Wife.	I	80
		Friend K.	II x	
2,906	I	Husband.	No pneumococcus.	
		Sister.	" "	
		Son.	" "	
2,908	I	Wife.	" "	
		Brother.	IV	
		Sister L.	No pneumococcus.	
		" A.	I	37
2,913	I	Mother.	No pneumococcus.	
		Brother.	IV	
2,917	I	Mother.	IV	
		Father.	No pneumococcus.	
		Brother C.	III	
		" B.	No pneumococcus.	
		Sister S.	IV	
		" N.	No pneumococcus.	

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				days
2,922	II	Wife.	II	70+
		Daughter I.	No pneumococcus.	
2,924	I	Wife.	" "	
		Daughter K.	" "	
		" L.	I	25
		" A.	No pneumococcus.	
		Son.	IV	
2,925	I	Wife.	III	
		Daughter S.	No pneumococcus.	
		" A.	" "	
		" R.	III	
		Son J.	I	25
		" M.	III	
2,926	II	Friend M.	III	
		" F.	No pneumococcus.	
		" L.	II	7
2,934	II	" R.	No pneumococcus.	
		" N.	IV	
		" H.	II x	
		" C.	No pneumococcus.	
		" K.	" "	
2,944	I	Mother.	I	32
		Father.	II b	
		Husband.	I	28
		Friend M.	No pneumococcus.	
		" McC.	IV	
		Daughter M.	No pneumococcus.	
		" H.	I and III	16
		Son.	No pneumococcus.	
		Sister.	I and II x	17
2,945	I	Mother.	I	7
		Father.	I	35+
		Brother I.	No pneumococcus.	
		" M.	" "	
		" C.	III and IV	
		Sister E.	I	9
		" Y.	No pneumococcus.	
		" S.	" "	
2,946	II	Wife.	" "	
		Son.	IV	

TABLE IV—*Concluded.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				days
2,952	I	Wife.	IV	
		Daughter.	No pneumococcus.	
2,954	I	Brother E.	" "	
		" J.	" "	
		Sister R.	" "	
		Cousin.	" "	
2,955	I	Wife.	I	21
		Son.	II x	
		Daughter.	I	5
2,968	I	Sister-in-law.	No pneumococcus.	
		Brother.	" "	
		Nephew J.	II b	
		" O.	IV	
2,971	II	Maid.	No pneumococcus.	
		Father.	" "	
2,976	II	Mother.	" "	
2,984	I	Brother.	IV	
		Nurse.	IV	
2,991	II	Sister.	IV	
2,996	I	Wife.	II x	
		Son.	No pneumococcus.	
M.M.	II	Wife.	IV	
		Mother-in-law.	IV	
		Nurse.	II x	

Summary.

Type.	No. of pneumonia households examined.	Households in which carriers were found.		Total contacts examined.	Positive contacts.	
			per cent			*per cent*
I.....	28	10	36	107	16	15
II.....	24	5	21	77	5	6
Total.....	52	15	28	184	21	11

Types of Pneumococci Obtained from Dust of Households in Which No Case of Pneumonia Had Occurred.

The presence of pneumococcus in dust has been a known fact for some time. Netter[4] in 1897 was the first to recover a definite pneumococcus from dust. Previous workers, notably Emmerich,[5] demonstrated the presence of Friedländer's bacillus in the dust of a room where there were many pneumonia patients. But at this early date the pneumococcus and Friedländer's bacillus were not clearly differentiated. The occurrence of the pneumococcus in dust has been lost sight of and very little significance has ever been attached to it. Its presence in dust has not been correlated with the occurrence of cases of pneumonia beyond a few casual references to the finding of pneumococcus in the dust of wards where there were many pneumonia patients.

In order to determine whether pneumococcus could be recovered with any regularity from dust the following study was carried out. The specimens of dust were collected as follows: A piece of paper was wrapped about a scrubbing brush which in turn was covered by a piece of cloth and then autoclaved. The dust was swept up with the sterile brush on to the paper which was then folded. This dust was mixed with sterile broth and 1 or 1.5 cc. of the mixture were immediately injected into the peritoneal cavity of a white mouse. Cultures were made from the heart's blood of the mouse.

In Table V are given the types of pneumococcus recovered from the dust of 62 rooms in which pneumonia had not occurred.

From these 62 specimens of dust, in 18 instances, or 29 per cent pneumococcus was recovered. In all but one instance these pneumococci belonged to those types which are normally found in the mouth. The specimen of dust from which the Type I pneumococcus was recovered came from a house where a known carrier of a Type I pneumococcus was visiting. From this study it is evident that pneumococcus can be easily recovered from dust. Furthermore, it appears that the strictly disease-producing types of pneumococcus, Types I and II, are not prevalent in dust when patients and healthy carriers of these types of pneumococcus are absent.

[4] Netter, L. D., *Compt. rend. Soc. biol.*, 1897, iv, series 10, 538.

[5] Emmerich, R., *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 167.

TABLE V.

Incidence of Pneumococcus in Dust from Rooms in Which Pneumonia Had Not Occurred.

Pneumococcus.	Incidence.	
		per cent
Type I	1	5.5
" II	0	0
" II a	0	0
" II b	4	22
" II x	3	16.6
" III	2	11
" IV	8	44.4
Pneumococcus present	18	
" absent	44	
	62	

Types of Pneumococci Obtained from Dust of Households in Which Pneumonia of Type I and Type II Had Occurred.

Table VI shows the incidence and types of pneumococcus found in 183 specimens of dust from households where cases of Type I or Type II pneumonia had occurred.

TABLE VI.

Incidence of Pneumococcus in Dust in the Presence of Pneumonia.

Pneumococcus.	Incidence.	
		per cent
Type I	25	33.78
" II	23	31.08
" II a	0	0
" II b	2	2.70
" II x	2	2.70
" III	2	2.70
" IV	20	27.02
Pneumococcus present	74	
" absent	109	
	183	

From 183 specimens of dust collected where cases of pneumonia due to pneumococcus of Type I or Type II had occurred, 74, or 40 per cent, showed pneumococcus. A Type I pneumococcus was found in 25 instances. In only one instance was a Type I pneumococcus recovered where a Type II pneumonia had occurred. This dust was collected 16 days after the patient had left his home. A Type II pneumococcus was recovered in 23 instances. In only one instance was a Type II pneumococcus found in the dust in the room where there had been a case of pneumonia due to Type I.

From this study it is evident that the highly parasitic pneumococci, Types I and II, are very prevalent in dust where cases of the same type of pneumonia have occurred. As the dust from only a comparatively small area of the floor was swept up, the organisms must have been present in a much larger proportion than these figures indicate.

In Table VII is presented a study of the types of pneumococcus obtained from the homes where a case of Type I or Type II pneumonia had occurred. As a rule, the dust was collected only from the patient's bedroom, but in some homes specimens were also obtained from the living-room and other bedrooms. Of 30 homes where a case of Type I pneumonia had occurred, in 13 instances, or 43 per cent, a Type I pneumococcus was recovered from at least 1 room. In 2 instances a Type I pneumococcus was recovered from 2 rooms. In all, 44 rooms were examined. From 15 of these rooms, or 34.08 per cent, a Type I pneumococcus was recovered. No Type II pneumococcus was recovered from a Type I home. Of 22 homes where a case of Type II pneumonia had occurred, in 13 instances, or 59 per cent, a Type II pneumococcus was discovered in the dust of at least 1 room. In 3 homes a Type II pneumococcus was recovered from 2 rooms. Of the 27 rooms which were examined, in 16, or 59 per cent, pneumococci of Type II were recovered. In only one instance was a Type I pneumococcus recovered from the dust of a Type II house. This occurred 16 days after the patient had left the house.

A comparison of Tables IV and VII shows that in the 28 Type I households in 3 instances the dust was positive, but no contact was found; in 2, the dust was negative, but a positive contact was present;

TABLE VII.

Incidence of Pneumococci in Dust from Homes Where a Case of Pneumonia Occurred.

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,746	I	Patient's bedroom.	0	6	No pneumococci.	Dirty and dark.
2,804	I	" "	3	6	" "	
2,814	I	" "	3	2	" "	Clean, but dark.
		" "	11		" "	
2,815	I	" "	1	4	I	Light, moderately clean.
		" "	12		I	
2,816	I	" "	1	6	I	Dark, fairly clean, poorly ventilated.
		" "	10		No pneumococci.	
2,821	I	" "	1	6	I	
		" "	10		No pneumococci.	
2,824	I	" "	14	3	" "	Very dirty and dark.
2,825	II	" "	1	1	III	Small, dark.
		" "	13		II	
		" "	49		No pneumococci.	
2,827	II	" "	3	3	II	Clean, light, and small.
		" "	10		II	
		" "	19		No pneumococci.	
2,834	II	" "	5	5	IV	Moderately clean, fairly well ventilated.
		" "	16		I	
2,852	I	" "	2	4	No pneumococci.	Large, clean, and light.
		" "	23		" "	
2,853	II	" "	8	2	" "	Very clean, light, well ventilated.
		" "	23		" "	
2,854	II	" "	4	3	" "	
		" "	14		" "	
2,858	I	" "	2	2	I	Moderately clean and light.
		" "	14		IV	
2,868	II	" "	2	4	No pneumococci.	Moderately light, well ventilated.
2,869	II	" "	3	2	" "	Clean.
		" "	18		" "	
2,874	I	" "	3	3	" "	Clean.
		" "	10		" "	
2,879	II	" "	2	2	" "	
		" "	15		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,880	I	Patient's bedroom.	2	3	IV	Moderately clean, well ventilated.
		" "	13		IV	
2,881	II	" "	2	6	IV	Dark, poorly ventilated.
		2nd "	2		IV	
		2nd "	13		IV	
2,883	I	Patient's "	5	4	No pneumococci.	Fairly clean but small.
		" "	15		" "	
2,885	II	" "	3	2	II	Clean.
		" "	13		No pneumococci.	
2,886	II	" "	3	4	II	Fairly clean but dark.
		" "	14		No pneumococci.	
2,890	II	" "	2	4	II	
		" "	15		No pneumococci.	
2,891	I	" "	1	3	" "	Clean.
		" "	23		" "	
2,892	II	" "	1	5	II	Moderately clean, fairly well ventilated.
		" "	23		II	
		" "	29		No pneumococci.	
2,896	II	" "	1	4?	II	Fairly clean, well ventilated.
		" "	24		No pneumococci.	
2,901	I	" "	2	5	I	Fairly clean, well ventilated.
		" "	25		I	
		" "	40		I	
		" "	50		I	
		" "	72		No pneumococci.	
		" "	92		" "	
2,906	I	" "	2	2	I	Large, clean.
		" "	21		No pneumococci.	
2,908	I	" "	3	5	I	Fairly clean.
		" "	18		No pneumococci.	
2,913	I	" "	2	5	" "	
		" "	13		" "	
2,917	I	Living-room.	8	6	II x	Fairly light.
		1st sick room.	8		No pneumococci.	Moderately clean.
		1st " "	22		" "	
		2nd " "	8		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,922	II	Living-room.	3	3	II	Fairly clean, moderately ventilated.
		"	22		No pneumococci.	
		"	37		" "	
		"	70		" "	
		Patient's bedroom.	3		II	
		" "	18		II	
		" "	37		II	
		" "	57		II	
		" "	70		No pneumococci.	
2,924	I	" "	3	5	" "	Fairly light and ventilated.
		" "	21		" "	
		Living-room.	3		" "	
2,925	I	Patient's bedroom.	1	5	" "	Small, dark, moderately clean.
		" "	16		I	
		" "	29		I	
		" "	45		No pneumococci.	
		Living-room.	2		" "	
		"	29		" "	
		"	45		" "	
2,926	II	Patient's bedroom.	6	4	" "	
		" "	6		" "	
		" "	20		II	
		" "	39		II	
		" "	55		No pneumococci.	
2,934	II	" "	5	4	" "	Clean.
		" "	14		" "	
2,944	I	1st "	3	5	IV	House thoroughly cleaned before second dust specimens were collected.
		1st "	16		No pneumococci.	
		1st "	53		II x	
		2nd "	3		I	
		2nd "	16		No pneumococci.	
		Mother's room.	3		I	
		" "	16		No pneumococci.	
		" "	53		II x	
		Living-room.	16		No pneumococci.	
2,945	I	"	3	5	I	Dirty, dark, but fairly ventilated.
		"	17		IV	
		"	35		IV	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,945	I	Patient's bedroom.	3		IV	
		" "	17		IV	
		" "	35		IV	
2,946	II	" "	1	3	No pneumococci.	
		" "	17		" "	
2,949	I	" "	24	8	" "	Fairly clean.
2,952	I	" "	3	2	" "	Light, clean, well ventilated.
		" "	21		" "	
		Living-room.	3		" "	
		"	21		" "	
2,954	I	"	5	4	" "	
		"	19		" "	
		Patient's bedroom.	5		" "	
		" "	19		" "	
2,955	I	" "	4	6	I	Moderately clean.
		" "	13		IV	
		" "	38		No pneumococci.	
		Living-room.	4		" "	
		"	13		I	
		"	38		No pneumococci.	
		"	52		" "	
2,968	I	"	3	5	IV	Fairly clean, fairly well ventilated.
		"	19		No pneumococci.	
		Patient's bedroom.	3		" "	
		" "	19		" "	
2,971	II	" "	2	4	II	Well ventilated and clean.
		" "	26		No pneumococci.	
		Living-room.	2		II	
		"	26		No pneumococci.	
2,976	II	"	3	3	" "	
		"	14		" "	
		"	41		" "	
		Patient's bedroom.	3		" "	
		" "	14		II	
		" "	41		No pneumococci.	
2,984	I	" "	7	5	" "	Dark, dirty basement.
2,991	II	" "	4	1	II	

TABLE VII—*Concluded.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of room.
			days			
2,991	II	Patient's bedroom.	26		No pneumococci.	
		Living-room.	4		II	
		"	26		No pneumococci.	
2,996	I	"	1	3	II b	
		"	15		No pneumococci.	
		Patient's bedroom.	1		" "	
		" "	15		" "	
		Another "	1		I	
		" "	15		I	
M.M.	II	Patient's present room.	0	?	II	
		Patient's present room.	0		No pneumococci.	
F.I.	I	Patient's bedroom.	1	?	I	

Summary.

Type.	No. of pneumonia households examined.	Households giving rise to positive dust.		Total No. of rooms examined.	Positive rooms.	
			per cent			*per cent*
I.....	30	13	43	44	15	34.08
II.....	22	13	59	27	16	59.25
Total.....	52	26	50	71	31	43.66

in 8, the dust and at least one individual showed a Type I pneumococcus; while in 15, a Type I pneumococcus was recovered neither from the dust nor from a member of the household. In the 22 Type II households, in 10 instances the dust alone was positive; in 1, an individual alone was positive; in 3, a Type II pneumococcus was recovered from both the dust and from a member of the household; and in 8, neither the dust nor an individual showed a Type II pneumococcus. In these 50 households in 13 instances the dust alone

was positive, while in only 3 was the dust negative in the presence of a positive contact.

Individuals or specimens of dust were studied from 54 households where a case of pneumonia due to a pneumococcus of Type I or Type II had occurred. Of the 30 Type I households, 14, or 46 per cent, showed either a positive human contact or a positive dust. From 24 Type II households 15, or 62 per cent, showed either a positive human contact or a positive dust. In other words, at the time of their admission to the hospital, the homes of over 53 per cent of the patients suffering from a pneumonia due to Type I or Type II were infected by a pneumococcus of the same type.

As a rule, the dust became negative before the contacts. But in two instances positive dusts were obtained after the carriers in the households had become negative. It is interesting that in the two homes which showed a positive dust for the longest time lived the two most persistent carriers. In Case 2,901, Type I, the dust was positive for 50 days, while the carrier had not become negative at the end of 80 days. In Case 2,922, Type II, the carrier was still positive at the end of 70 days and the dust was positive on the 57th day.

Two Pneumonia Epidemics.

Through the courtesy of the State Department of Health and the local health officer of Rochester, N. Y., I have had an opportunity of studying two epidemics of pneumonia in institutions.

One epidemic occurred in a boys' asylum. At the time of the epidemic there were over 200 boys in this institution, but the 6 cases of pneumonia were limited to the boys occupying two of the four dormitories. 3 boys in each dormitory developed pneumonia; from the sputum of 3 of the 6 boys a Type I pneumococcus was recovered, from another a Type II x, and from a 5th a Type IV; from 1, no pneumococcus was obtained. The absence of Type I pneumococcus from 3 cases may be due to the fact that this study was not made until the patients were convalescent. The sputum of the other 56 boys who slept in the two dormitories was studied. From the saliva of 6, or 10 per cent, a Type I pneumococcus was isolated.

Three specimens of dust were taken at random from each of the

two dormitories. As these rooms were very clean this dust had to be swept out from between the chinks of the floor boards. One specimen of dust from each dormitory showed a Type I pneumococcus. Of the other four specimens two showed a Type IV, and from two no pneumococcus was obtained. Six specimens of dust from the vacant room used as a ward failed to show a Type I pneumococcus, but a pneumococcus of Type II and a pneumococcus of Type II b were recovered.

In this epidemic 50 per cent of the cases of pneumonia were found to be due to Type I pneumococcus; from 10 per cent of the healthy contacts and from the dust a Type I pneumococcus was recovered.

The other epidemic occurred in the Rochester State Hospital for the Insane. Here six cases of pneumonia occurred among the inmates of one ward of over 200 persons. A Type I pneumococcus was recovered from four of the patients, but the other two patients died before the type of infecting pneumococcus was determined. Each of the patients who died had shared a double room with one of the patients who showed a Type I pneumococcus. A Type I pneumococcus was also recovered from the dust of one of these two bedrooms. The saliva of 148 inmates of this ward was studied. In five instances a Type I, and in one a Type II pneumococcus were isolated. In all, nine specimens of dust were examined. In one instance a Type I, in one, a Type III, and in three, a Type IV pneumococcus was found.

In this epidemic six cases of pneumonia occurred in the ward of an institution. A Type I pneumococcus was recovered from four of the six patients, from 2 per cent of the healthy contacts, and from the dust.

Related Cases of Pneumonia.

Several instances which seem to be contact infections or infections from the same source have been studied. In the first instance a mother daily visited her son who was critically ill with a Type I pneumonia. The mother contracted a bad cold and developed pneumonia due to a Type I pneumococcus 11 days after her son was taken ill. Another case was that of an actor who was admitted to the hospital with a Type II pneumonia. The next day an actor who

shared the same dressing-room developed a Type II pneumonia. Before the end of the week an electrician at the same theater became ill with pneumonia. He also showed a Type II pneumococcus. In a third instance Miss H. (type of pneumonia not determined) was taken to a private hospital by Mrs. A. who developed a Type II pneumonia in a few days. Mr. B. helped to nurse Mrs. A. and shortly fell sick with a Type II pneumonia. In another instance a patient who had just recovered from a Type I pneumonia left the hospital March 9. The next day he went to see his brother who had developed a Type II pneumonia. On March 12 this patient, who had just recovered from a Type I infection, developed a pneumonia due to a Type II pneumococcus. The following case suggests the possibility of an infection by a healthy carrier. A patient was admitted to the hospital suffering from pneumonia due to Pneumococcus Type I. Specimens of sputum were obtained from the other members of the household. One 5 year old daughter was found to be a Type I pneumococcus carrier; the other two members of the household were negative. The dust from the room the patient had previously occupied in this house also showed a Type I pneumococcus. The little girl was sent to board with friends while her mother was in the hospital. She spent 3 days with the first family and then went to visit in the Bronx. 6 days after she left, a child in the home where she had visited came down with pneumonia due to Pneumococcus Type I. Specimens of sputum from the other members of this household were negative, but from the dust in the sick child's room a pneumococcus of Type I was isolated. The daughter of the original patient visited in the Bronx for 10 days. No cases of pneumonia developed in this home and the sputum of the members of this family as well as the dust failed to show the presence of Pneumococcus Type I. The child next went to visit friends in Brooklyn. Although the sputum from the members of this household were negative, from the dust a Type I pneumococcus was recovered.

DISCUSSION.

The results of the work detailed in this paper confirm the previous observations of Dochez and Avery on the occurrence of healthy carriers of disease-producing types of pneumococcus. Consideration

of the results of study over a period of years of the types of pneumococcus inducing lobar pneumonia shows that in the majority of instances infection is due to organisms belonging to Type I or Type II. The minority of cases, on the other hand, are due to infection with pneumococcus of Types III and IV.

Comparison of the types of pneumococcus obtained from the mouth secretions of normal persons with those isolated from individuals with lobar pneumonia shows the existence of two general classes of organisms. One of these, which consists of Types I and II, occurs only in association with disease. The other, which includes Types III and IV and the atypical Type II organisms, also causes pneumonia but these organisms are commonly found in normal healthy mouths. Rarely Types I and II have been found in the mouth secretions of normal individuals who give no history of association with cases of pneumonia. On the other hand, organisms of Types I and II have been found in 11 per cent of normal individuals who have been in intimate association with a case of pneumonia of the same type.

Although the presence of pneumococcus in dust has been known for some time, little significance has been attached to it. The results of this work show that pneumococcus can be easily recovered from dust. The types of pneumococcus found reflect accurately the pneumococcal flora of the mouth of the members of these households. Pneumococcus of Types I and II is rarely found in dust except where a case of pneumonia due to the same type of pneumococcus has occurred. In view of the ease with which dust can be disseminated it is not surprising that in a few instances a Type I or Type II organism was recovered from the dust which did not correspond to the type of pneumococcus producing the disease.

The occurrence of these disease-producing types of pneumococcus in the dust suggests the possibility that air-borne infection may play a part in the production of pneumonia. On the other hand, the mere presence of the disease-producing types of pneumococci in the mouth will not initiate disease. But if a susceptible individual comes in intimate contact with a case of pneumonia there is grave danger of his contracting the disease.

These facts suggest the following conclusions concerning the epidemiology of lobar pneumonia. Infection with pneumococcus of

Types I and II must be regarded as dependent upon either direct or indirect contact with a previous case of lobar pneumonia due to the same type of organism. These types of infection are either acquired by direct contact with a previous case of pneumonia, by association with a healthy carrier of one of these types of pneumococcus, or possibly by an air-borne infection from dust which has been infected. Infection with the sputum types of pneumococcus, namely Types III and IV and the atypical strains of Type II, may be autogenic, or due to the acquisition by the individual of one of these types to which he is especially susceptible.

SUMMARY.

1. Pneumococci of Type I and Type II are responsible for the majority of the cases of lobar pneumonia.

2. Among the pneumococci found in the mouths of healthy individuals Type IV predominates, Type III is frequent, and atypical organisms of Type II are occasionally found.

3. Healthy persons intimately associated with cases of lobar pneumonia may harbor in their mouth secretions the highly parasitic pneumococcus of Types I and II.

4. Occasionally a carrier of Type I or Type II pneumococcus is encountered in whom it is impossible to trace any contact with an infected patient.

5. From the dust of homes where cases of pneumonia due to Types I and II have occurred, pneumococci of the same type may be recovered.

THE PRODUCTION OF ANTIPNEUMOCOCCIC SERUM.

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Ever since the demonstration in 1891 by Foa and Carbone,[1] Emmerich and Fowitzky,[2] and G. and F. Klemperer[3] of the immunizing properties of the serum of animals rendered immune to pneumococcus, this serum, modified in various ways, has been employed to a greater or less extent in the treatment of pneumonia in man.

Besides the employment of sera prepared in various ways in small series of cases, there has been an extensive employment of the sera prepared according to the directions of Pane,[4] Washbourn,[5] and more lately Römer[6] and Neufeld and Händel.[7, 8] In this country at least six commercial houses which sell biologic products have for some years been producing immune serum for the treatment of pneumonia and pneumococcus infections. It is impossible to know how extensively these sera have been employed, certainly to a sufficient extent to render their production profitable.

In spite of all that has been written concerning the theoretical principles involved in the preparation of antipneumococcic serum, and in spite of all the reports of its therapeutic application which have appeared, it is very difficult to learn from the literature on the subject exactly how these sera have been prepared or standardized.

[1] Foa, P., and Carbone, T., *Gazz. med. Torino*, 1891, xlii, 1.

[2] Emmerich, R., and Fowitzky, A., *Münch. med. Woch.*, 1891, xxxviii, 554.

[3] Klemperer, G., and Klemperer, F., *Berl. klin. Woch.*, 1891, xxviii, 833, 869.

[4] Pane, N., *Centr. Bakteriol., Ite Abt.*, 1897, xxi, 664.

[5] Washbourn, J. W., *Brit. Med. J.*, 1897, i, 510.

[6] Römer, P., *Arch. Ophth.*, 1902, liv, 99; Experimentelle und klinische Grundlagen für die Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea (Ulcus serpens), Wiesbaden, 1909.

[7] Neufeld, F., and Händel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 159.

[8] Neufeld and Händel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 166, 1293.

Without this knowledge we can have no accurate starting-point from which to proceed toward improvements in methods of production.

In view of these facts and also since an antipneumococcic serum is now being prepared by The Rockefeller Institute for Medical Research and used in the Hospital of this institution, and since serum made in a similar manner is now being prepared by a number of commercial houses and by several public health laboratories for wider distribution, it has seemed important that an accurate description of the methods employed by us in its production be published, together with a brief discussion of the theoretical basis for the methods employed.

Theoretical Considerations Concerning the Production of Antipneumococcic Serum.

In spite of a general belief to the contrary, all kinds of animals, even the most susceptible, may be rendered actively immune to pneumococcus infection, by the previous injection of non-lethal doses of living pneumococci or even by the injection of the dead bodies. Indeed, the substances of the bacteria which give rise to the immune reaction are very resistant to various chemical and physical agents, and a review of the literature shows that it is possible to produce active immunity with a great variety of antigens prepared from the bacteria. The degree of immunity, however, differs, depending upon the procedures employed.

The serum of the actively immunized animals, in many cases, possesses protective and curative power, the degree of this power depending somewhat on the height of the active immunity, but not invariably or regularly so. It may be stated, however, that animals whose serum is protective and curative are always actively immune. On the other hand, an animal may itself be fairly highly immune without the serum containing any immune bodies that we can demonstrate, and without its having any demonstrable protective action. For instance, we have immunized rabbits so that they have successfully withstood 0.1 cc. of a living culture, of which 0.000001 cc. killed the control, without the blood showing a trace of immune bodies or any protective power. Quite frequently animals whose serum shows only moderate grades of immunizing value may be actively resistant

to enormous doses. The fact that active immunity and serum-immunizing power do not run exactly parallel does not necessarily indicate that active immunity in pneumococcus infection differs fundamentally from passive immunity, but suggests this possibility. At any rate, it suggests that in the reaction of resistance to, or recovery from, infection there may be other factors concerned than the humoral ones. Nevertheless, with present knowledge of serum therapy our effort must be confined to the production of a serum having a high content in demonstrable antibodies and a high protective value. The chief kinds of antibodies which can be demonstrated in this immune serum are agglutinins and opsonins or bacteriotropins, and the kind of protective power meant is that which is exhibited when a very small amount of the serum is injected into a susceptible animal such as the mouse, simultaneously with, or within a few hours following, the injection of a large dose of virulent culture, which alone would cause rapid death of the animal. These are the properties of the serum which are at present tested to indicate its therapeutic strength.

It would lead us far from our present purpose, were we to discuss here at length the mode of action of the serum, for the discussion would necessarily involve a consideration of the mechanism of resistance and recovery. It is not believed that the action of the immune serum is entirely dependent either on its power of causing agglutination (Bull[9]) or on its bacteriotropic power (Neufeld and Rimpau[10] and Neufeld and Händel[7, 8]), though these properties may play important parts. They, however, are susceptible of quantitative estimation. The protection of small animals undoubtedly reproduces more accurately the part which the serum plays in recovery from natural infection, as seen in the human patient, but even here the conditions are not identical.

At the present time, however, the effectiveness of the serum seems to be parallel to its content in the antibodies we have mentioned, and especially to its protective power tested as we have described. We believe the production of antipneumococcic serum that may be accurately standardized is of fundamental importance. Whether all theoretical qualifications are fulfilled is not so essential.

[9] Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

[10] Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 283.

Specificity.

The primary requisite for the serum is that it is specific. Everything stated above concerning both active and passive immunity is true only if the bacterium acted upon, either in antibody tests *in vitro*, or in protection tests *in vivo*, is identical with the organism used in producing the serum. This does not merely mean that the bacteria shall belong to the same species, for it is now generally known that different races of the same species of bacteria may show differences in their antigenic properties, even without differences in their cultural characteristics. These differences have heretofore, however, been considered differences merely in degree and mainly in lesser degree. To overcome the difficulties which this fact puts in the way of the production of immune serum for practical purposes, recourse has been had to the method of immunizing an animal with many different strains, producing a polyvalent serum. This method, for instance, has been made use of to a considerable extent in the production of antimeningococcic serum. Although certain races of meningococci differ from each other quite markedly in their immunological properties, yet they all have common characteristics which render a sharp differentiation difficult, if not impossible. The conditions as regards meningococci at present seem to be somewhat as follows. If we employ the Ehrlich nomenclature, each strain of meningococcus is endowed with a large number of kinds of receptors which we may designate by the letters of the alphabet. In one large group of strains of meningococcus *a* receptors predominate; in another group *b* receptors predominate, etc. In smaller groups other receptors, such as *k*, *l*, *m*, or *n* receptors, are most numerous. However, even in the first two groups, the *a* and *b* receptors are not exclusively present or even overwhelmingly predominating. Indeed each of the different races possesses practically all the different kinds of receptors but in greatly varying degrees.

Under these conditions, in order to produce a serum which will be active against all the different races it is necessary to choose and employ for immunization a large number of races in order that the entire receptor "spectrum" shall be covered as uniformly as possible. It seems that it is possible to do this fairly well as far as meningococcus is concerned.

The conditions as concerns pneumococcus are similar, though not identical. From agglutination experiments Kindborg[11] decided that all strains of pneumococci were immunologically distinct. Neufeld first brought evidence of group relationships and this fact has been elaborated and extended by the work in The Rockefeller Institute Hospital and elsewhere. Dochez and Gillespie[12] have demonstrated that the pneumococci obtained from cases of pneumonia occurring in this country belong in four large groups. The immunological characteristics of the organisms of three of these groups are very specific. The conditions therefore among pneumococcus are different from those obtaining among meningococcus. It is difficult to demonstrate by immunological methods that pneumococcus of Type I has any immunological characteristics (or receptors in the Ehrlich nomenclature) which are common to all pneumococci. Complement fixation tests, however, seem to show that the pneumococci of the different types do possess some common characters, at least these tests are not absolutely specific as regards the different types (Hanes[13]) and the antihemolytic reactions of specific sera against the hemolytic toxins derived from different types of pneumococci (Cole[14]) are not absolutely specific. So too the extremely active agglutinating immune sera produced by the injection of pneumococci of specific types may have slight degrees of effectiveness against certain strains of other types (Blake[15]). But in general the different types of pneumococci have a high degree of specificity as shown by protective action and by agglutination. Active immunity to the different types also seems very specific. This type specificity, which was only lately recognized, is of fundamental and primary importance in considering the practical application of immunity to therapy in this disease. Since nothing was known of this group specificity before the observations of Neufeld were made, it is very difficult to draw any conclusions from the observations or from the results of their practical application which were made in this field before that time. Where identical strains were

[11] Kindborg, A., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 197.
[12] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.
[13] Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.
[14] Cole, R., *J. Exp. Med.*, 1914, xx, 346.
[15] Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 67.

used throughout experiments, the conclusions of course were valid, but in the absence of knowledge of the importance of using only these strains, it is probable that in many cases this procedure was not strictly adhered to.

The work done in the Hospital of The Rockefeller Institute, both experimentally and clinically, indicates that immune serum against Type I infection is more effective than that against any other of the specific types. Indeed the results obtained both here and elsewhere indicate that this serum has great therapeutic value when it is employed in the treatment of cases due to the same type of pneumococcus. On the other hand, the observations which have so far been made with sera of the other types do not show that these produce distinct therapeutic effects. It is possible, of course, that improvements in the method of production or mode of application of these other types of serum may lead to more satisfactory results. For the present, however, we believe that the general practical application of this form of therapy should be confined to Type I cases, and this may readily be done since the type of infection in the individual case may easily be determined by the methods described elsewhere (Blake[15]). We also think it important at present that the commercial production of antipneumococcic serum for therapeutic purposes be confined to that effective against Type I infection. This paper aims to present the best method for manufacturing this serum and is based on the experience we have gained through the immunization of a very large number of rabbits, several goats, and four horses to Type I pneumococcus, four horses to Type II pneumococcus, one to Type III pneumococcus, and one to both Type I and Type II pneumococci, or ten horses in all.

Animals to Be Employed for Immunization Purposes.

For practical reasons small animals cannot be used to supply serum for therapeutic purposes, especially if the large amounts of serum which are now considered necessary are to be used.

In the earlier therapeutic tests, such as those of G. and F. Klemperer and Foa and Carbone, rabbits were used for obtaining the small amounts of serum used. Later Washbourn used ponies for immunizing purposes. Pane used a cow and an ass. Neufeld used horses in the preparation of his serum.

Römer[6] has laid stress on the importance of combining the sera prepared by immunizing a number of different animals. His reason for this is a purely theoretical one; namely, that all the different antigenic elements contained in a complex structure such as the pneumococcus may not find suitable receptors in any one animal. With present knowledge it seems that we may disregard this consideration.

We believe that for practical reasons it is best to use horses. Our experience is not sufficient to enable us to have a definite opinion as to the kind of horses best to choose for this purpose; apparently, however, draft horses are superior to the lighter grades and to the more finely bred animals. Joint injuries, indeed injuries of all kinds, undoubtedly contribute to the localization of pneumococcus and consequent focal infection. The highly strung, nervous horses are more liable to these injuries and infections. These accidents delay immunization. As regards antibody response, there has been marked individual variation, but the difference has not been associated with any obvious distinction in type of horse. In rabbits, with which our experience is much larger, there is very great individual difference. It is far more important in the production of this kind of serum than in the production of antitoxic sera that the horses should be perfectly sound and healthy, especially without old joint injuries.

Site of Injection.

Neufeld has laid stress on the value of intravenous injections. We have also employed this method since local reactions are thereby avoided. With dead cultures the local reactions are usually not severe or important; where living cultures are employed, however, they may be violent and abscess formation not infrequently occurs. It is felt, moreover, that by intravenous injections the bacteria are brought more rapidly and quickly and with less opportunity for change into contact with widely distributed cells, the reaction with which is supposed to result in the immunity response.

Choice of Antigen.

Use of Living or Dead Cultures.—As we have previously stated, the production of active immunity is attended with little difficulty and the form of antigen, so far as we know, is not of great importance,

but when we come to the question of the production of humoral immunity, especially of the highest grade, this factor may be of the greatest importance. It has generally been assumed on theoretical grounds that to produce the highest grade of humoral immunity it is necessary to use living cultures. It seems likely that all the various constituents of the bacterial cell and even some products of growth, which may be very labile, give rise to specific antibodies. Therefore to obtain the most varied and complete reaction it has been thought necessary to employ the living organisms, which may, for a short time at least, grow in the body and produce or give rise by their dissolution to certain labile metabolic substances which may stimulate antibody formation. Most of the antipneumococcic sera which have been prepared, at least of late years, have been produced by the injection first of dead, then of living organisms. The general acceptance of this method is probably due in part to what is known concerning immunity in tuberculosis. Here, as is well known, the only immunity that is effective has been produced by the injection of living organisms, by producing a mild tuberculous infection. One must be careful, however, in applying what is known concerning one type of infection directly to another, without due consideration. As knowledge of infection increases it becomes more and more evident that each disease must be considered independently. As we shall show later, it is possible that in the production of antipneumococcic serum the living organisms are not so important as they have previously been considered.

Virulence of Organisms Employed.—Pneumococci may rapidly lose their virulence for animals when grown in an artificial medium. This loss of virulence, however, may not be uniform for all kinds of experimental animals. Moreover, the virulence of pneumococci which have long been grown on artificial media may be increased by passing repeatedly through experimental animals. Here again the increase of virulence may not be uniform for all the varieties of animals. It is possible, for instance, to obtain a given strain which is very virulent for guinea pigs, with little virulence for rabbits. Even for animals as closely related in their susceptibility to pneumococcus infection as the rabbit and mouse, it is possible to have races which are highly virulent for one with little virulence for the other.

It is manifest, therefore, that we can judge only very imperfectly of the virulence of a given race for man by determining its virulence for a susceptible animal, such as the mouse or rabbit. Whether in the production of an immune serum it is important for the organisms employed to be highly virulent or not is not known. Neufeld and his associates[7, 10] have laid stress on the employment of virulent cultures, believing that the action of immune serum is to neutralize exactly those receptors of the bacterium upon which virulence depends. They state that they have proved experimentally that immunity cannot be produced with avirulent races, but give no protocols. Races virulent for mice have been employed in all our immunizing studies and we have no observations concerning immunization with avirulent races. If virulence of the organism used as antigen is important, what we have just stated concerning virulence for different species of animals becomes of great significance. It will be remembered that mice are used in all our tests of immunity. The fact that a serum is protective for mice would indicate that it had been produced by a race of pneumococci virulent for mice, but the test might give us little information relative to its protective power for man. We have therefore come to believe, on these purely theoretical grounds, that the immunization of horses should be carried on with organisms which have not been long under artificial cultivation since their isolation from the human body. These organisms are practically always virulent for mice, so that where they are employed, the test of the protective power of the serum in mice is probably a good test for protective and curative power in man. In order to have a culture which has not long been removed from the human body always ready for use, advantage may be taken of an observation made by Heim[16] and confirmed by Neufeld and Händel[7] and abundantly corroborated by us; namely, that when pneumococci are preserved in pieces of dried tissue or blood they remain viable for a very long time, and also retain their virulence undiminished. For preserving small amounts of culture the spleens of infected mice, dried and kept in a vacuum, are most satisfactory. For preserving large amounts of culture from human patients, it is well to inoculate a rabbit with blood or other infected material. After infection has reached a maximum grade,

[16] Heim, L., *Z. Hyg. u. Infectionskrankh.*, 1905, l, 123.

the rabbit is bled and the blood is spread in thin layers in Petri dishes and dried. To obtain a fresh culture at any time all that is necessary is to inoculate a little of this dried material into the peritoneal cavity of a mouse and later make a fresh culture from the heart's blood. For immunizing purposes a fresh culture obtained in this way should be prepared every 1 or 2 weeks. This precaution in obtaining cultures should be taken not only when the live bacteria are to be injected, but also when dead organisms are to be employed.

Method of Growing Organisms Used for Injection.—The use of bacteria grown in broth and separated by centrifugalization from the medium in which they are grown was first employed by Neufeld[17] who believed that the substances formed in the medium during growth are not useful but indeed harmful. We also think that it is not necessary to use the fluid in which the bacteria are cultivated. In this belief we differ from Wadsworth[18] whose observations apparently show that the serum produced with whole cultures is more effective than that produced by the injection of the bacteria alone. His experiments, however, are not entirely conclusive and in the absence of any method of accurately titrating this increased efficacy, it does not seem advisable to employ the whole culture. Moreover, the injection of the whole culture adds greatly to the difficulty of the immunizing process. As is well known, even fresh bouillon is toxic and after bacterial growth has taken place it is still more toxic. In using whole cultures, therefore, one is much restricted as to the amount that can safely be injected. When the amount of culture to be injected reaches a large size, the technical difficulties and time required in centrifugalizing the cultures become considerable. We have attempted to overcome these difficulties by growing the pneumococci on blood glucose agar in flasks and washing off the surface growth in salt solution, using the emulsion so obtained, without centrifugalizing. For certain theoretical reasons, moreover, we thought this method might be of advantage. In our experience, however, the method of growing in bouillon and centrifugalizing still proves the most satisfactory. In our earlier work the organisms were obtained by growing in broth,

[17] Neufeld, F., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 54.
[18] Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.

centrifugalizing, and washing once in salt solution, and then resuspending in salt solution. In our later work, however, we have not thought it necessary to wash, but have merely centrifugalized and then made an emulsion of the sediment in salt solution.

For obtaining a satisfactory growth in bouillon the reaction of the medium is of great importance. Pneumococci grow best in a medium the reaction of which is 0.3 to 0.5 per cent acid to phenolphthalein. If the reaction is more acid than this, a satisfactory growth may not be obtained. We have added no sugar, serum, or other enriching substance to the medium employed.

Size and Spacing of Doses.

In our first studies we employed the method which has been largely employed by others, making the injections every 7 to 8 days. This is the method generally employed in immunization and has developed from the observation that the most efficient stimulus can be applied at the time when the immunological response to the preceding dose is most active. Grades of high immunity have been thought to increase in a step-like manner, each increment being added to that previously present. It is again not certain, however, that antipneumococcus immunity obeys the same laws as other forms of immunity in which this step-like rise occurs. Certain of our observations to be mentioned later indicate that even when the injections are made at the period of greatest activity, instead of a rise in the immunity, there may be a fall, especially if the dosage is too large. After a primary immunity had been obtained by weekly injections of dead cultures, live organisms were injected, beginning with small doses, *i.e.*, the bacteria from 2 to 5 cc. of bouillon culture, and the succeeding weekly injections were gradually increased in size up to the bacteria contained in 1 or even 2 liters of culture. Neufeld speaks of injecting doses of living pneumococci as large as the bacteria obtained from 1,500 cc. of culture in the horse, and from 3,500 cc. in the ass. He injected doses of dead organisms as large as the bacteria contained in 9 liters of culture. In our experience this method of immunization is attended with many disadvantages. It has required 6 to 8 months to bring horses up to the desired grade of immunity. That these very large doses are not necessary is shown

by the following protocol of a horse in which the attempt was made to produce an effective serum by using only small doses of culture (Table I).

TABLE I.

Horse 1. Immunized to Pneumococcus Type I.

Date.	Injection.	Tests of serum.
1916		
Feb. 2	3,250 units of tetanus antitoxin subcutaneously.	
" 4	Bacteria from 25 cc. of culture, killed by heating.	
" 11	" " 50 " " " " " "	
" 18	" " 75 " " " " " "	
" 26	" " 100 " " " " " "	
Mar. 3	" living, from 2.5 cc. of culture.	
" 11	" " " 5.0 " " "	
" 19	" " " 10.0 " " "	
" 29	" " " 20.0 " " "	
Apr. 3	" " " 40.0 " " "	
" 15	" " " 80.0 " " "	
" 22		Agglutination:* complete. Protection:† 0.1 cc., D.; 0.01 cc. S.
" 22	Bacteria, living, from 120 cc. of culture.	
May 7		Agglutination:* complete. Protection:† 0.1 cc., S.

* These tests were made before we commenced the routine accurate titration of the agglutination strength.

† In the protection tests each of the mice received 0.2 cc. of serum simultaneously with a graduated dose of culture, both given intraperitoneally. The figures given indicate the amount of culture added. D. indicates died; S., survived. For brevity, only the highest dose with which recovery took place is given. In all cases the control animals receiving 0.000001 cc. of culture alone died.

It is true that the results obtained with this horse were unusually good, better than any we have since been able to obtain with a similar method. They indicate, however, that the large doses which we had been using were not necessary, that equally good results could be obtained with much smaller amounts of culture.

Modification and Improvements in the Methods of Immunization.

Over a year ago we undertook experiments to determine whether or not animals could be immunized more rapidly than had been done in the past, and also whether it might not be possible to obtain a higher grade of immunity than we had previously observed, and finally to determine for ourselves whether the use of living organisms is necessary in producing humoral immunity.

To determine the best methods of immunization a large number of rabbits was immunized in various ways and the development of immunity studied. Certain observations which had been made indicated that the process might be hastened by more frequent injections of antigen than had previously been used.

In 1900 Dean[19] showed that in the production of diphtheria antitoxic immunity the administration of the toxin at 3 day intervals gave very successful and practical results. Daily doses of antitoxin have been administered with good results when other methods have failed.[20] In 1908 Fornet and Müller[21] showed that precipitating sera could be produced very rapidly by three daily injections of antigen, bleeding on the 12th day. Bonhoff and Tsuzuki[22] confirmed these observations and Tsuzuki[23] showed that by a similar method a rapid production of typhoid-agglutinating serum could be produced. Similar observations have been made by Gay and his assistants.[24]

Flexner and Amoss[25] have employed a similar method in the production of antidysenteric serum, injecting live cultures on 3 successive days, with excellent results. In the same way the method of three daily injections has been employed by Amoss and Wollstein[26] in the production of antimeningococcic serum.

In the production of antidysenteric and antimeningococcic serum stress has been laid on regulating the size of the dose so that, following each inoculation, a febrile reaction shall be obtained. It occurred to us that the choice of three daily doses had been made more or

[19] Dean, G., *Tr. Path. Soc. London*, 1900, li, 15.

[20] Personal communication from Dr. Theobald Smith.

[21] Fornet, W., and Müller, M., *Z. biol. Techn. u. Method.*, 1908–09, i, 201.

[22] Bonhoff, H., and Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 180.

[23] Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 194.

[24] Gay, F. P., *Ergebn. Immunitätsforsch. exp. Therap., Bakteriol. u. Hyg.*, 1914, i, 231.

[25] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

[26] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

less arbitrarily and that possibly daily doses administered over a longer time might be still more efficacious in producing a satisfactory result, especially since this method had proved of value in the production of diphtheria antitoxin. That this method is indeed of much value is seen from the results of the experiments given below. The experiments need not be described here in detail. Certain slight variations due to external causes were made in individual instances, but most of the animals were treated about as follows.

Series 1.—These animals received weekly intravenous injections of large amounts of bacteria obtained by centrifugalization of broth cultures, which were then killed by heating for 1 hour at 56°C. The size of the doses varied from the bacteria contained in 100 cc. of broth to those contained in 500 cc.

Series 2.—These animals received intravenous injections of very small amounts of bacteria killed by heat as in the above experiment; the doses were given daily for 7 days, then an interval of 7 days was allowed to elapse, and a second series of injections was given, etc. The bacteria in the individual doses varied from those contained in 1 cc. of broth to those contained in 2 cc.

Series 3.—These animals received combined doses of immune horse serum and living cultures intravenously, beginning with 1 cc. of serum plus 0.1 to 0.5 cc. of culture. This dose was repeated every day for 3 days, then 7 days were allowed to elapse, and a second series of these combined doses was given, this time using slightly larger doses of culture. After an interval of another week, combined doses using still larger amounts of culture were given, etc.

Series 4.—These animals received varying sized doses of pneumococci killed by the addition of 0.5 per cent carbolic acid. The bacteria were centrifugalized from broth cultures, taken up in salt solution, the carbolic acid was added, and the mixture kept at 37°C. over night. As in the preceding series, the injections were given intravenously on 3 successive days, then an interval of a week was allowed to elapse, and the second series given. The number of bacteria in the individual doses varied from those contained in 2 cc. of broth to those in 50 cc. of broth. Some animals received large weekly doses subcutaneously.

Series 5.—These animals received injections of an antigen prepared as follows. The bacteria were grown in broth, centrifugalized, washed, and taken up in a very small amount of salt solution, and this emulsion was added to a large amount of acetone. The sediment which formed was centrifugalized at once, dried in a vacuum, and after 12 hours in the vacuum, taken up in salt solution and thoroughly shaken. From this somewhat viscid translucent fluid further dilutions in salt solution were made. The injections in these rabbits were made once a week, and the doses varied from an amount of antigen representing the bacteria contained in 5 cc. of broth to one representing the bacteria contained in 220 cc. of broth. The small doses were given intravenously, the larger ones subcutaneously.

In addition to the above, a small series of rabbits received injections of antigen prepared by freezing and grinding the bacteria, and another small series received injections of bacteria dissolved in bile.

In studies such as these it must be borne in mind that individual rabbits immunized in exactly the same way may show quite marked variations in their immunity response. Slight differences, therefore, in results obtained in small series of animals are not of significance.

It would lead us too far to attempt to analyze in detail the results in the various tests or to publish the protocols. One definite fact stands out from these studies. Uniformly the results following the injection of small doses of killed culture given daily over a period of 7 days followed by 7 days of rest were excellent (Series 2). This is in marked contrast to the results obtained by the injection of large doses of killed cultures given at intervals of a week (Series 1). Serum from these animals showed little or no evidence of immunity. This is strikingly shown in Table II.

TABLE II.

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
			Rabbit 1.		
1916		*gm.*			
May 27	1	1,670	Bacteria from 250 cc. of culture, killed by heating.		
	7 day interval.				
June 4	9	1,400	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 14	19	1,300	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 24	29	1,270	Bacteria from 250 cc. of culture, killed by heating.		
	6 day interval.				
July 1	36	1,150		Undiluted, 0	0.001 cc., D.

TABLE II—*Continued.*

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
			Rabbit 2.		
1916		*gm.*			
May 28	1	1,520	Bacteria from 1 cc. of culture, killed by heating.		
" 29	2		Bacteria from 1 cc. of culture, killed by heating.		
" 30	3		Bacteria from 1 cc. of culture, killed by heating.		
" 31	4		Bacteria from 1 cc. of culture, killed by heating.		
June 1	5		Bacteria from 1 cc. of culture, killed by heating.		
" 2	6		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 10	14	1,650	Bacteria from 1 cc. of culture, killed by heating.		
" 11	15		Bacteria from 1 cc. of culture, killed by heating.		
" 12	16		Bacteria from 1 cc. of culture, killed by heating.		
" 13	17		Bacteria from 1 cc. of culture, killed by heating.		
" 14	18		Bacteria from 1 cc. of culture, killed by heating.		
" 15	19		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 23	27	1,180	Bacteria from 1 cc. of culture, killed by heating.		
" 24	28		Bacteria from 1 cc. of culture, killed by heating.		
" 25	29		Bacteria from 1 cc. of culture, killed by heating.		
" 26	30		Bacteria from 1 cc. of culture, killed by heating.		
" 27	31		Bacteria from 1 cc. of culture, killed by heating.		
	8 day interval.				
July 6	40	1,100		1:200 +	0.2 cc., D.
				1:400 0	0.1 cc., S.

As the protocols show the serum of Rabbit 1 injected weekly with large doses of culture showed no agglutinating power and only moderate protective action. On the other hand, the serum of Rabbit 2 which was treated over practically the same period of time but received very small daily doses showed high agglutinating strength, positive in a dilution of 1: 200, and high protective power. These results were most surprising and striking, especially as all of the eight rabbits inoculated with the small daily doses showed an extraordinarily prompt and active response, while all of the eight rabbits inoculated with the large doses showed very little or no response in the same period of time.

The studies of other methods of immunization gave little information. The attempts to produce immunity with combined doses of serum and culture (Series 3) gave unsatisfactory results. The experiments of this series were undertaken because by this method live cultures could be injected at the very beginning of the immunization. Moreover, Theobald Smith[27] and von Behring[28] have shown the possibility of immunizing with combined doses of toxin and antitoxin. Besredka[29] and others have shown the possibility of immunizing against typhoid with sensitized cultures. Levy and Aoki[30] claim to have produced immunity to pneumococci with great rapidity (in 6 hours) by the injection of sensitized bacteria killed with carbolic acid. However, in view of what is now known concerning possible dissociation of pneumococcus antigen and antibody (Gay and Chickering[31]), it is possible that what the latter writers observed was not active immunity but slight grades of passive immunity. In any case, the grade of immunity produced was slight. Our experiments yielded no evidence in favor of the combined injection of culture and immune serum. Nevertheless, it is possible that other modifications of the method might yield better results, especially as we made no effort to balance accurately the amounts of culture and immune serum employed.

[27] Smith, Theobald, *J. Med. Research*, 1907, xvi, 359.
[28] von Behring, E., *Deutsch. med. Woch.*, 1913, xxxix, 873.
[29] Besredka, cited by Gay.[24]
[30] Levy, E., and Aoki, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 435.
[31] Gay, F. P., and Chickering, H. T., *J. Exp. Med.*, 1915, xxi, 389.

The serum of the rabbits of Series 4 inoculated with cultures killed by carbolic acid indicated no considerable grade of immunity. There is apparently no advantage to be gained in employing antigen prepared in this way. Levy and Aoki,[30] however, have reported the production of immune serum in dogs by the use of this method; but the grade of immunity, according to our standards, was slight.

Finally, the experiments in Series 5 and those in which frozen and ground bacteria and bacteria dissolved in bile were employed simply offer observations in the use of bacterial antigenic substances produced in other ways.

Neufeld[32] and Vetrano[33] have also employed bile extracts of pneumococci for immunizing purposes, only, however, in the production of active immunity. Others have employed still other artificial methods of treating the bacteria. G. and F. Klemperer[3] used glycerol extracts and Wadsworth[18] attempted immunization with cultures precipitated with alcohol and dissolved in water.

These and our own observations with artificially produced antigens show that slight grades of immunity can be produced by antigens prepared by various methods. The antigenic substance seems to be highly resistant. However, neither the observations of others nor our own indicate tha[illegible]ese methods are especially useful in extracting the antigenic substa[illegible] or in rendering it more effective. Indeed in all instances the immunity reaction resulting from the employment of antigens prepared in these ways was less intense than that following the injection of heat-killed bacteria. One fact emerges from these experiments, however, though in a less striking way than from the observations previously mentioned; namely, that small doses repeated frequently are much more effective than large doses given at longer intervals. The very large doses seem to have a definite repressing action on the development of antibodies.

These observations led us to immunize a series of four rabbits with small daily doses to determine the exact time of appearance of the immune properties in the serum. Table III is a typical protocol of one of the rabbits.

[32] Neufeld, *Z. Hyg. u. Infectionskrankh.*, 1900, xxxiv, 454.
[33] Vetrano, G., *Centr. Bakteriol., 1te Abt., Orig.*, 1909, lii, 275.

TABLE III.

Rabbit 3.

Date.	Weight.	Injection of vaccine.	Agglutination tests.	Protection tests.
1916	*gm.*	*cc.*		
May 29	1,950		0	0.0001 cc., D. 36 hrs.
				0.00001 " " 36 "
				0.000001 " " 36 "
" 29	1,950	1*		
" 30		1		
" 31	1,900	1		
June 1		1		
" 2		1		
" 3		1		
" 9	1,910		1:1 ++	0.1 cc., D. 16 hrs.
			1:10 0	0.01 " " 79 "
				0.001 " S.
				0.0001 " "
" 11		1		
" 12		1		
" 13	1,900	1		
" 14		1		
" 15	1,750	1		
" 16		1		
" 23			1:1 ++	0.1 cc., S.
			1:10 ++	0.01 " "
			1:20 +	0.001 " "
" 24		1		
" 25	1,750	1		
" 26		1		
" 27	1,900	1		
" 28		1		
" 29	1,850	1		
July 5	1,950			
" 7			1:100 ++	0.2 cc., D. 20 hrs.
			1:200 +	0.1 " S.
			1:400 0	0.01 " "

* In these experiments the material for injection was prepared at the beginning of the experiment and kept on ice. 150 cc. of an 18 hour broth culture of Pneumococcus Type I was centrifugalized and the sediment washed once in salt solution. The sediment was taken up in 10 cc. of salt solution and heated ½ hour at 56°C. Cultures were sterile. The emulsion was kept on ice and, after shaking, a small amount was removed and diluted to original volume before each injection. The injections were made intravenously.

The experiment shows that it is possible by this method of immunization to produce a high grade of immunity within 6 weeks; even within 4 weeks a considerable grade of immunity is present. The serum from each of the four rabbits showed a high agglutinating titer and a constant protective power against 0.01 to 0.1 cc. of culture. Two of the rabbits lost slightly in weight, one remained stationary, and one actually gained. They showed no other ill effects.

Experiments have also been made to show whether similar or better results could be obtained by injecting animals daily with small doses over longer periods than 7 days. Four rabbits were given small daily doses for 14 days and then bled and the serum was tested on the 5th and 10th days following the last injection. The serum of these

TABLE IV.

Horse 2. Immunized to Pneumococcus Type I.

Date.	Injection.	Agglutination test.	Protection tests.
1916			
Jan. 27	1,500 units of tetanus antitoxin subcutaneously.		
" 29	Bacteria from 50 cc. of culture, killed by heating.		
" 30	" " 50 " " " " " "		
" 31	" " 50 " " " " " "		
Feb. 1	" " 50 " " " " " "		
" 2	" " 50 " " " " " "		
" 3	" " 50 " " " " " "		
" 10	" " 50 " " " " " "		
" 11	" " 50 " " " " " "		
" 12	" " 50 " " " " " "		
" 13	" " 50 " " " " " "		
" 14	" " 50 " " " " " "		
" 15	" " 50 " " " " " "		
" 23	" " 50 " " " " " "		
" 24	" " 50 " " " " " "		
" 25	" " 50 " " " " " "		
" 26	" " 50 " " " " " "		
" 27	" " 50 " " " " " "		
" 28	" " 50 " " " " " "		
Mar. 8		1:200 +	0.1 cc., S. 0.1 " D. 0.01 " S.

animals has shown very slight agglutinating and protective power, so that this modification seems to be of no advantage.

The results of the rabbit experiments have led us to try the method of immunization described above in horses. The result obtained in one horse treated in this manner is given in Table IV.

The results in this and other horses have shown that it is possible in this way to produce a very high grade of primary immunity with a great saving in time and without danger to the animal. The dosage has been arbitrarily chosen. It is possible that with further experience and more carefully regulated dosage it may be possible to produce even higher grades of immunity in this way. It may even be possible by this method to obviate entirely the use of live cultures.

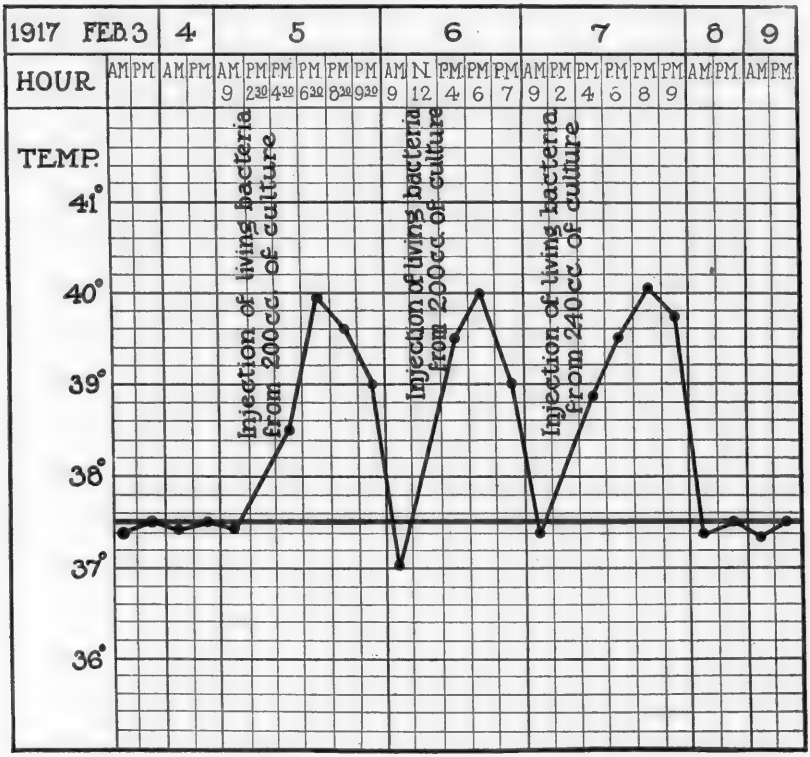
What has been attempted so far, however, has been the production of a primary immunity in the quickest possible time and with the least loss in horses.

TABLE V.

Horse 2, Undergoing Immunization Since January 29, 1916. Last Bleeding, 11 Liters, on May 15, 1917.

Date.	Injection.	Protection tests.
1917		
May 26	Bacteria from 100 cc. of culture, killed by heating.	
" 27	" " 100 " " " " " "	
" 28	" " 100 " " " " " "	
" 29	" " 100 " " " " " "	
" 30	" " 100 " " " " " "	
June 1	" " 100 " " " " " "	
" 9	" " 100 " " " " " "	
" 11	" " 100 " " " " " "	
" 14	" " 100 " " " " " "	
" 15	" " 100 " " " " " "	
" 16	" " 100 " " " " " "	
" 17	" " 100 " " " " " "	
June 25	Blood test.	0.01 cc., D.
July 6	Bacteria, living, from 100 cc. of culture.	
" 7	" " " 130 " " "	
" 8	" " " 160 " " "	
" 13	Blood test.	0.2 cc., S.

The problem now confronting us is to determine the method by which horses having an established primary immunity may be brought up to the highest possible level and kept there. In order to do this, when dead cultures have failed, it may be necessary to resort to living


1917 FEB 3
4
5
6
7
8
9
HOUR
AM PM AM PM AM PM PM PM PM PM AM N PM PM PM AM PM PM PM PM PM AM PM AM PM
9 2½ 4½ 6½ 8½ 9½ 9 12 4 6 7 9 2 4 6 8 9
TEMP.
41°
40°
39°
38°
37°
36°
Injection of living bacteria from 200cc of culture
Injection of living bacteria from 200cc of culture
Injection of living bacteria from 240cc of culture

TEXT-FIG. 1. Temperature curve of Horse 3 injected with living Pneumococcus Type I.

cultures. An observation on one of our horses makes this probable (Table V).

In this horse, whereas repeated small daily doses of killed culture failed to bring the serum up to full strength, three moderate sized doses of living culture caused the serum to acquire maximum power.

This result has been seen on numerous occasions and it is probable that in most horses living cultures must finally be employed. When injections of living cultures are made we now employ the method described by Flexner and Amoss,[25] administering three doses in amounts sufficient to produce a moderate febrile reaction. A typical curve is shown in Text-fig. 1. So far as pneumococcus immunity, however, is concerned, the necessity for producing febrile reactions is not established. Neufeld believed that so far as agglutinating sera are concerned the power depends not so much on the height of the immunity as on the intensity of the last reaction through which the animal had passed. Therefore he thought it advisable to inject as large amounts as possible without killing the animal. The observations we have made do not support this point of view. In our experience with horses a violent reaction is not always followed by a marked immunity response or increase in agglutinating power of the serum; indeed the opposite is frequently the case. For the present, however, in giving live cultures it is probably better to be guided in the size of the dose by the febrile reactions. We think, however, that large doses should be avoided even though the febrile reaction is slight.

Typical Method of Immunization Based on Previous Observations.

As a result of our observations and those of others we now believe that immunization should be carried out according to the method as at present employed by us, which is briefly given below.

Having obtained a sound, fairly heavy horse, it is first given a glanders test. At present the complement fixation test is used for this purpose. A specimen of 20 cc. or more of blood is obtained before any treatment is given and is kept for use in control tests. The immunization is then carried out as follows. All injections are made intravenously, employing for this purpose a Luer syringe. To avoid any accidental injury to the vein it is well to have the needle attached to the syringe with a small piece of rubber tubing. The culture used for injection, whether living or dead, should be one highly virulent for mice, 0.000001 cc. killing regularly, and it should have gone through very few passages in animals or on artificial media since removal from the human patient. The method for keeping the cultures is described above (page 545). In preparing the material for injection, both living and dead, the organisms are grown on beef peptone broth, reaction 0.3 to 0.5 per cent acid to phenolphthalein. Cultures about 12

to 15 hours old are preferable as at this time maximum growth is present, with a minimum of autolysis (Chesney[34]). The cultures should contain about 200 to 300 million bacteria per cc. The culture is centrifugalized until the supernatant fluid is clear. With the large centrifuge employed by us this requires about 20 to 30 minutes. The supernatant fluid is then poured off and the sediment is taken up in a small amount of sterile salt solution.

If the organisms are to be injected alive, the emulsion in salt solution is not made until just before injection, so that autolysis and death may not take place. If the organisms are to be injected dead, the emulsion is placed in a tube in a water bath and kept at 56°C. for ¾ hour. For the daily injections a considerable amount of the emulsion, after killing, is prepared and kept on ice. This may be employed for all the injections in the series of 6 to 7 days. We think, however, a fresh emulsion should be prepared each week. For each injection the dilution of the fluid should be such that the volume injected is about 20 cc.

The following course of injections is now carried out. Every day for 6 days an amount of the emulsion of killed pneumococci containing the bacteria from 50 cc. of the bouillon culture is injected. An interval of 7 days is allowed to elapse and then a second series of daily injections, of the same size, is made. Again an interval is allowed to elapse and on the 6th day a specimen of blood is obtained for testing. Tests are made at once for agglutinating and protective power. This requires several days.

If the serum causes agglutination in a dilution of 1: 200 and is of standard protective value, 0.2 cc. regularly protecting a mouse against 0.1 cc. of a virulent culture, bleeding may be carried out at once; that is, on the 10th to 12th day following the last injection. As a matter of fact, we have never seen the titer of the serum after this amount of treatment to be so high. Consequently it has been our practice, and we advise, that 8 to 10 days after the last injection of the second series of dead bacteria, injection of live organisms be commenced. These injections are given on successive days. The first injection should consist of the bacteria contained in 20 cc. of the original culture. The temperature is taken every 2 hours for 8 to 10 hours following each of the injections of live cultures. If the temperature reaction is only moderate, not over 40.5°C., an injection of the bacteria from 40 cc. of culture is given on the following day. If the reaction from this is only moderate, the dose is again doubled on the following day and the bacteria from 80 cc. of culture are injected. As before stated, so far as pneumococci are concerned it is difficult to regulate the dosage entirely by the febrile reaction obtained. If the reaction is very severe, of course the dose is made smaller than those mentioned. On the other hand, even though the reaction is very slight, we do not advise giving more than the amounts stated. 6 days after the last injection another specimen of blood is obtained for testing. If the serum is of the standard strength, bleeding can now be done. If it is still too weak, a second

[34] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

series of injections of living bacteria is made. These injections should consist of the bacteria from 100 cc., 150 cc., and 200 cc. of broth on the 3 days respectively. Again, the size of the doses may need to be somewhat modified, on account of the severity or lack of febrile reaction. Certain horses may require still further injections of live cultures but this is exceptional. In any case, we think the injections should be made in series of three, given on successive days, with 7 day intervals between each series, and from our present standpoint we believe that the size of the injection should never be greater than the bacteria from 300 to 400 cc. of broth. Following the bleeding, it is well to allow the horse to remain quiet for 3 or 4 days. Then a series of three injections of living cultures, 50, 80, and 100 cc., is again given. After a week the serum is again tested and if of standard strength bleeding may again be done on the 10th day following the last injection.

CONCLUSIONS.

In the production of immune serum for therapeutic purposes strict attention must be paid to the immunological specificity of the bacteria used for immunization. At present the only serum of which the therapeutic value has been proven is that effective against Type I pneumococcus infection. This serum should have agglutinating power for Type I pneumococcus and should have the power of protecting mice against large amounts of virulent culture. Experiments have shown that for producing the primary immunity most rapidly several series of small doses of dead cultures should be given, the injections being made daily for 6 to 7 days, followed by a week in which no injections are made. To produce the highest type of immunity probably living organisms are required. These should be given in moderate doses daily for 3 days, with an interval of a week between each series of injections. By following accurately the methods described, horses may be made to produce rapidly a high grade of specific serum. The observations so far made indicate the importance of employing small doses of culture frequently repeated in this form of immunization.

STUDIES ON ANTIBLASTIC IMMUNITY.*

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The following studies were undertaken with the purpose of throwing more light, if possible, on the influence exerted by immune serum on the metabolic activities of bacteria. It has been shown that under certain experimental conditions immune serum possesses the property of inhibiting or markedly retarding the metabolic functions of various microorganisms. Bacterial metabolic processes are generally considered to be enzymotic in nature. It is not known whether they take place primarily at the surface of the bacterial cell or within the cell body. It is probable, however, that exogenous metabolism occurs to a considerable extent in preparing nutrient material for absorption.

Evidence for this may be found in the studies by Cole[1] on the formation of methemoglobin by the pneumococcus. The property of immune serum which enables it to inhibit the metabolic activities of bacteria is considered to act at the surface of the bacterial cell and to be antienzymotic in nature. The term antiblastic immunity has been applied as descriptive of the phenomenon.

Von Dungern[2] has shown that the liquefaction of gelatin by *Staphylococcus pyogenes aureus* is inhibited by antistaphylococcus immune serum; Ghéorghiewsky[3] showed that immune serum inhibits pigment formation by *B. pyocyaneus*; and Ascoli[4] considered that the action of antianthrax serum was in part dependent upon the inhibition of capsule formation by *B. anthracis* with consequent suppression of the metabolic activities of the organism. More recently Dochez and Avery[5] have shown that antipneumococcus immune serum retards the growth of the pneumococcus and inhibits or markedly delays the fermentation of inulin and the splitting of protein by the pneumococcus.

* This work was done under the tenure of a William O. Moseley Travelling Fellowship from Harvard University.

[1] Cole, R., *J. Exp. Med.*, 1914, xx, 363.

[2] von Dungern, *Centr. Bakteriol., Ite Abt.*, 1898, xxiv, 710.

[3] Ghéorghiewsky, *Ann. Inst. Pasteur*, 1899, xiii, 298.

[4] Ascoli, A., *Centr. Bakteriol., Ite Abt., Orig.*, 1908, xlvi, 178.

[5] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

EXPERIMENTAL.

The majority of the experiments reported below deal with the antiblastic properties of antipneumococcus serum. A few experiments have been done with antistaphylococcus serum.

Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Immune Serum.

Characteristic metabolic functions of the pneumococcus are the fermentation of inulin and litmus milk, the splitting of protein, and the transformation of oxyhemoglobin to methemoglobin. The influence which immune serum exerts on these functions of the pneumococcus has been studied. The experiments of Dochez and Avery have been repeated and extended and their experimental results confirmed in that under certain conditions antipneumococcus serum has been found to possess the property of inhibiting or retarding the metabolic activities of pneumococci. The conclusions drawn from our experiments as to the mechanism of this inhibitory property of antipneumococcus serum, however, differ from those of Dochez and Avery.

Experiment 1. Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.—In Table I is shown one of a series of experiments on the rate of growth of the pneumococcus in homologous and heterologous antipneumococcus serum and in normal horse serum. The amount of growth was determined by the plate method.

This experiment shows that an apparent inhibition of growth occurs in homologous antipneumococcus serum as compared with that in normal serum during a period of 3 hours, that a retardation of growth occurs up to 6 hours, but that by 24 hours an abundant growth is present in the immune serum as well as in the normal serum. It furthermore appears that there is some retardation of growth of Type I pneumococcus by the heterologous immune serum, a phenomenon which does not occur with Type II pneumococcus. These results agree with those of Dochez and Avery. It is felt, however, that the interpretation of such results as indicative of actual retardation of growth is open to serious objection. It is clear that the apparent inhibition of growth by homologous serum must in large part

TABLE I.

Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.

Culture 0.00000001 cc.	Serum 0.2 cc.	No. of colonies.			
		Immediately.	After 3 hrs.	After 6 hrs.	After 24 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	61	54	80	Confluent.
" " I	Antipneumococcus Serum Type II	50	45	12,000	"
" " I	Normal horse serum.	37	284	22,000	"
Pneumococcus Type II	Antipneumococcus Serum Type I	31	69	6,200	Confluent.
" " II	Antipneumococcus Serum Type II	43	42	1,860	"
" " II	Normal horse serum.	29	59	7,750	"

be due to agglutination. Furthermore, it is well known that during the first hours of growth of the pneumococcus chain formation occurs to a considerable extent. This is a variable phenomenon which is difficult to control. For these reasons it seems hazardous to conclude from the experiments that immune serum actually retards the growth of the pneumococcus and quite as probable that the inhibition indicated by the colony counts is more apparent than real.

Experiment 2. Inhibition of the Fermentation of Inulin and Litmus Milk by Antipneumococcus Serum.—The power of antipneumococcus serum to inhibit the fermentation of inulin and litmus milk by the pneumococcus has been tested in a considerable series of experiments, one of which is shown in Table II.

This experiment demonstrates that the fermentation of litmus milk by the pneumococcus is markedly delayed in the presence of the homologous immune serum. The fermentation of inulin is retarded by antipneumococcus serum in a similar manner. In the tubes containing the homologous immune serum the organisms grow in agglutinated masses at the bottom of the tube, while in the tubes containing the heterologous or normal serum they grow diffusely. To demonstrate clearly inhibition of fermentation it was found necessary to use extremely small amounts of pneumococcus culture (not more than 0.00001 cc.) in inoculating the media. With the use of

TABLE II.

Inhibition of the Fermentation of Litmus Milk by Antipneumococcus Serum.

Litmus milk 4 cc. +		Incubation at 37°C.			
Culture 0.000001 cc.	Serum 1 cc.	24 hrs.	48 hrs.	72 hrs.	5 days.
Pneumococcus Type I	Antipneumococcus Serum Type I	−	−	±	+
" " I	" " " II	++	++	++	++
" " I	Normal horse serum.	++	++	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	±+	++	++	++
" " II	" " " II	−	±	±	+
" " II	Normal horse serum.	++	++	++	++

++ indicates complete acidification and coagulation; ±+, acid and incomplete coagulation; +, acid and beginning coagulation; ±, acid and no coagulation; ±, slight acidification and no coagulation; −, no acidification or coagulation.

larger amounts retardation of fermentation was very slight or did not occur at all. In a series of experiments to determine the maximum amount of culture that could be used satisfactorily in demonstrating the antiblastic action of immune serum in preventing the fermentation of inulin, it was frequently noted that in the immediate vicinity of the agglutinated pneumococci at the bottom of the culture tube there was acidification of the medium indicated by change of the litmus indicator to red, while the upper portion of the medium remained unchanged. This observation suggested the possibility that the apparent antiblastic action of antipneumococcus serum was in reality due merely to agglutination of the organisms at the bottom of the culture tube and their consequent inability to come into intimate contact with the medium as a whole, this not being the case in the presence of heterologous immune or normal serum. If this is so, to conclude from such experiments as those cited above that immune serum possesses a specific antiblastic action would hardly be justified. Further experiments on this point are given below.

Experiment 3. Inhibition of Methemoglobin Formation by Antipneumococcus Serum.—The transformation of oxyhemoglobin to methemoglobin is a characteristic metabolic function of the pneumococcus which lends itself readily to *in vitro* experiments. A large series of experiments has been done in order to determine under what conditions antipneumococcus serum will inhibit this

activity of the pneumococcus. In general, the results have been similar to those obtained in the case of litmus milk and inulin. To demonstrate inhibition, it was found essential to use very small amounts of pneumococci in inoculating the hemoglobin solution-serum mixtures and to allow the cultures to incubate 24 hours. One of these experiments is shown in Table III, which demonstrates that under these conditions homologous antipneumococcus serum inhibits the transformation of oxyhemoglobin to methemoglobin by the pneumococcus. Heterologous immune serum and normal horse serum possessed no inhibitory properties.

TABLE III.

Inhibition of Methemoglobin Formation by Antipneumococcus Serum.

Hemoglobin solution* 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	−	−
" " I	" " " II	++	++
" " I	Normal horse serum.	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++
" " II	" " " II	−	±
" " II	Normal horse serum.	++	++

++ indicates complete transformation of oxyhemoglobin to methemoglobin; ±, slight methemoglobin formation; −, no methemoglobin formation.

* Hemoglobin solution = 2 cc. of a 5 per cent suspension of washed rabbit corpuscles + 8 cc. of distilled water + 30 cc. of 0.85 per cent salt solution.

With the use of larger amounts of culture it was found that the formation of methemoglobin was not inhibited by immune serum, but took place rapidly in the immediate vicinity of the agglutinated pneumococci and gradually diffused upward throughout the whole medium. These results, as in the case of inulin, suggested that the apparent inhibition which occurs when minute amounts of culture are used is due to inability of the agglutinated pneumococci to come into intimate contact with the medium as a whole.

Experiment 4. Inhibition of the Proteolytic Functions of the Pneumococcus by Antipneumococcus Serum.—The growth of pneumococcus in a serum broth medium is attended by an increase in the amino-acid content of the medium. Dochez and Avery[5] believe it probable that the pneumococcus effects a splitting of pro-

tein before absorption and that the increase in amino-acid content of the medium represents the excess of protein split over that used up in the process of growth. If this hypothesis is accepted, the increase in amino-acid produced by the growth of the pneumococcus might serve as a measure of the proteolytic activity of the pneumococcus in a given culture, the amount of increase being directly proportional to the total amount split. The possibility should be pointed out, however, that the amount of amino-acid increase might be inversely proportional to the metabolic activities of the pneumococcus, a small amino-acid increase indicating a greater utilization of the total amount of protein split, and *vice versa*.

The experiment recorded in Table IV shows the increase in amino-acid nitrogen when the pneumococcus was grown in broth containing antipneumococcus serum as compared with the increase in the presence of normal horse serum. The increase in amino-acid was determined by the method of Van Slyke.[6]

TABLE IV.

Inhibition of the Proteolytic Activities of the Pneumococcus by Antipneumococcus Serum.

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37° C.
Culture 0.000002 cc.	Serum 2 cc.	
		mg.
Pneumococcus Type I	Antipneumococcus Serum Type I	0.031
" " I	" " " II	0.103
" " I	Normal horse serum.	0.106

If the increase in amino nitrogen content of the medium is a direct measure of the proteolytic activities of the pneumococcus, it is apparent that the homologous antipneumococcus serum exerted a marked inhibitory action on this metabolic function of the pneumococcus. Since the pneumococcus growth in the tube containing homologous immune serum was in agglutinated masses at the bottom of the tube in contradistinction to the diffuse growth in the other tubes, it seemed possible that this factor might be responsible for the apparent inhibition of proteolytic activity rather than any true antienzymotic property of the serum. A further experiment to determine the influence of this factor is presented below.

Experiment 5. Antiblastic Properties of Exhausted Immune Serum.—A series of experiments was done to determine whether immune serum exhausted of its

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121; 1915, xxiii, 407.

agglutinin and precipitin content by repeated exposure to killed pneumococci still retained any demonstrable antiblastic properties. In Table V is shown one of these experiments from which it will be seen that immune serum exhausted of its agglutinin and precipitin content possesses no power to inhibit the formation of methemoglobin by the pneumococcus. Identical results have been obtained with respect to the fermentation of inulin and litmus milk. From these experiments it is evident that the specific antiblastic action of immune serum, if such exists, is removed from the serum at the same time that the agglutinins and precipitins are removed by treatment with dead pneumococci.

TABLE V.

Antiblastic Properties of Exhausted Antipneumococcus Serum.

Hemoglobin solution 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	−	−
" " I	Exhausted " " " I	++	++
Pneumococcus Type II	Antipneumococcus Serum Type II	−	±
" " II	Exhausted " " " II	++	++

Experiment 6. Relation of Agglutinins to Antiblastic Immunity.—From the results obtained in the foregoing experiments the suggestion arose that the apparent antiblastic action of antipneumococcus serum was in some way closely related to agglutination of the bacteria. The following experiment was done to determine more accurately this relationship. Equal parts of pneumococcus cultures and increasing serum dilutions were mixed in small tubes and incubated for 2 hours at 37°C. 1 cc. of hemoglobin solution was then carefully added to each tube so as not to disturb the agglutinated pneumococci and the tubes were incubated for 1 hour at 37°C. The results are shown in Table VI.

This experiment shows that the antiblastic action of the serum bears a definite relationship to the degree of agglutination of the pneumococci. In the tube containing the 1:25 serum dilution agglutination was complete, the pneumococci having entirely settled to the bottom of the tube, and complete inhibition of methemoglobin formation occurred. With increasing dilutions of serum agglutination and sedimentation were progressively less complete and formation of methemoglobin rapidly took place in the immediate neighborhood of the agglutinated pneumococci, the upper portion of the medium

TABLE VI.

Relation of Antiblastic Immunity to Agglutination.

Culture 0.5 cc.	Serum 0.5 cc.	Agglutination after 2 hrs. at 37° C.		Methemoglobin formation after 1 hr. at 37° C.
Pneumococcus Type I	Antipneumococcus Serum Type I (1: 25)	++	1 cc. of hemoglobin solution added to each tube.	−
" " I	Antipneumococcus Serum Type I (1: 50)	$\frac{-}{++}$		++
" " I	Antipneumococcus Serum Type I (1: 100)	+		− ++
" " I	Antipneumococcus Serum Type I (1: 200)	±		− ++
" " I	Antipneumococcus Serum Type I (1: 400)	−		++
" " I	Antipneumococcus Serum Type II (1: 25)	−		++
" " I	Normal horse serum (1: 25)	−		++

$\frac{-}{++}$ indicates methemoglobin formation in the immediate vicinity of the agglutinated pneumococci, the upper portion of the medium remaining unchanged.

from which the bacteria had settled out remaining unchanged. This experiment indicates that the antiblastic property of antipneumococcus serum probably depends in large measure, if not entirely, upon its ability to agglutinate the pneumococci and thereby prevent their coming into intimate contact with the medium as a whole.

By growing pneumococci in a serum-hemoglobin solution agar medium, agglutination of the pneumococci may be prevented. Under these conditions antipneumococcus serum was found not to inhibit methemoglobin formation. An objection may be raised to these experiments, however, because it is not certain that in a solid medium the immune serum is in sufficient concentration in the immediate vicinity of the pneumococcus colonies to exert a demonstrable antiblastic action.

Experiments 7 and 8. Relation of Antiblastic Immunity to the Ability of Bacteria to Come into Intimate Contact with the Medium.—It seemed probable from many of the previous experiments that the antiblastic action of antipneumococcus serum was due to inability of the agglutinated bacteria to come into sufficiently

intimate contact with the medium as a whole to enable them to produce the characteristic changes in the medium. Under these conditions the metabolic activities of the pneumococci could exert their influence only in the immediate vicinity of the agglutinated bacteria and complete change of the medium could take place only as gradual diffusion occurred. This is exactly what did occur when moderately large amounts of pneumococci were used, the reaction in the presence of homologous immune serum being merely retarded rather than completely inhibited. When minute amounts of pneumococci were used growth had probably ceased before diffusion could take place and apparently complete inhibition resulted.

In order to obviate this difficulty, two sets of experiments have been carried out. The first consisted in running a parallel series of tubes, one of which was shaken, the other not. The second group of experiments consisted in a parallel series of cultures, one in test-tubes, the other in small flasks so that the agglutinated bacteria would be brought into much more intimate contact with the medium as a whole, which was in a thin layer at the bottom of the flasks, than could possibly occur in test-tube cultures. The effect of shaking is well demonstrated in the experiment shown in Table VII.

TABLE VII.

Effect of Shaking on the Inhibition of Methemoglobin Formation by Antipneumococcus Serum.

2 hrs. at 37°C.			Incubation at 37°C.				
Culture 0.5 cc.	Serum 0.5 cc.			10 min.	30 min.	1 hr.	Shaken 10 min.
Pneumococcus Type I	Antipneumococcus Serum Type I	1 cc. of hemoglobin solution in each tube.	Unshaken.	−	−	−	++
" " I	Antipneumococcus Serum Type I		Shaken.	++	++	++	

Suitable control tubes showed that normal horse serum did not inhibit methemoglobin formation and that shaking in itself did not change oxyhemoglobin to methemoglobin. While complete inhibition of methemoglobin formation took place in the unshaken tube in the presence of homologous antipneumococcus serum, in the tube that was shaken so that the agglutinated clumps of bacteria were more or less broken up and diffused throughout the medium no inhibition occurred.

Further experiments illustrating this point were made with two strains of *Streptococcus viridans* which, like the pneumococcus, transforms oxyhemoglobin to methemoglobin. Strain 1 normally grew diffusely in serum-free bouillon, but like many streptococci grew in granular clumps at the bottom of the culture tube in the presence of serum. By growing this streptococcus in tubes containing antipneumococcus serum, it was possible to inhibit completely for 24 hours the formation of methemoglobin as shown in Table VIII, demonstrable methemoglobin being present only after gradual diffusion of the medium took place.

TABLE VIII.

Inhibition of Methemoglobin Formation by Streptococcus viridans by Antipneumococcus Serum.

Hemoglobin solution 1 cc. +		Methemoglobin formation.		Character of growth.
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.	
Streptococcus viridans 1	Antipneumococcus Type I Serum	−	+	Sediment.
" " 1	Antipneumococcus Type II Serum	−	+	"
" " 1	Normal horse serum.	±	++	" and diffuse.
" " 1	Bouillon 1 cc.	++	++	Diffuse.

This inhibition cannot be attributed to any specific immunity principle in the serum and is more probably brought about by the growth of the streptococci at the bottom of the culture tube and their consequent inability to act on the medium as a whole.

Streptococcus viridans 2 normally grew as a sediment in bouillon and produced methemoglobin slowly as the growth gradually extended up the sides of the tube and diffusion of the medium took place. By shaking a culture of *Streptococcus viridans* 2 so that the organisms were in continual intimate contact with the whole medium it was found that the transformation of oxyhemoglobin to methemoglobin took place rapidly and was complete by the end of 6 hours,

at which time the unshaken control tubes failed to show any trace of methemoglobin (Table IX).

TABLE IX.

Effect of Shaking on Methemoglobin Formation by Streptococcus viridans.

Materials used.	Methemoglobin formation after incubation at 37°C.	
	3 hrs.	6 hrs.
Streptococcus viridans 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (shaken)	±	++
Streptococcus viridans 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (unshaken)	−	−
Hemoglobin solution 1 cc. + bouillon 1.5 cc. (shaken)	−	−
" " 1 " + " 1.5 " (unshaken)	−	−

It is evident from this experiment that the rapid formation of methemoglobin by this streptococcus depended upon its ability to grow in intimate contact with the whole medium.

Comparison of the inhibitory power of immune serum in parallel series of test-tube cultures and flask cultures confirmed the results of the shaking experiments. It was found that while marked retardation of metabolic function as measured by the change in the medium occurred in the test-tube in the presence of homologous immune serum, no inhibition occurred in the flask cultures in which the agglutinated bacteria were able to come into intimate contact with the medium as a whole. This is clearly shown in the experiments recorded in Table X.

From these experiments it is evident that antipneumococcus serum exerted no inhibitory action on the fermentation of litmus milk or on the splitting of protein by the pneumococcus when the organisms were grown in flask cultures, in striking contrast with the results in the test-tube cultures. Identical results were obtained with respect to the fermentation of inulin.

These two groups of experiments seem to throw considerable doubt on the theory that antipneumococcus serum possesses the property of inhibiting the metabolic activities of the pneumococcus by virtue of a specific antiblastic immunity principle. They rather indicate

TABLE X.

Relation of the Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Serum to the Ability of the Pneumococcus to Act on the Medium as a Whole.

Fermentation of Litmus Milk.

Litmus milk 4 cc. +		Test-tube cultures.			Flask cultures.		
Culture 0.000001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 24 hrs.	After 48 hrs.	After 72 hrs.
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++	++	++	++	++
" " II	Antipneumococcus Serum Type II	−	±	±	++	++	++
" " II	Normal horse serum.	++	++	++	++	++	++

Digestion of Protein.

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37°C.	
Culture 0.000002 cc.	Serum 2 cc.	Test-tubecultures.	Flask cultures.
		mg.	*mg.*
Pneumococcus Type I	Antipneumococcus Serum Type I	0.031	0.169
" " I	" " " II	0.103	0.120
" " I	Normal horse serum.	0.106	0.154

that the inhibition which occurs under certain experimental conditions is in reality due to agglutination of the pneumococci by the immune serum and their consequent inability to come into intimate contact with the medium as a whole.

Inhibition of the Metabolic Activities of Staphylococcus pyogenes aureus by Antistaphylococcus Serum.

Characteristic metabolic functions of *Staphylococcus pyogenes aureus* are the liquefaction of gelatin, the formation of a golden pigment, and the transformation of oxyhemoglobin to a magenta-colored unstable compound, which is probably reduced hemoglobin.

A limited series of experiments has been carried out to determine the ability of antistaphylococcus serum to inhibit or retard these metabolic activities of *Staphylococcus pyogenes aureus*. The staphylococcus used in the experiments was obtained by blood culture from a case of staphylococcus septicemia and an autologous immune serum was prepared by the immunization of a rabbit with this staphylococcus.

Experiment 9. Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.—The liquefaction of gelatin is a characteristic metabolic function of *Staphylococcus pyogenes aureus*. In a series of experiments to determine the inhibitory action exerted by antistaphylococcus serum on this activity of the staphylococcus it was found that complete inhibition occurred. One of these experiments is shown in Table XI. Incubation of the culture tubes was carried out at 20°C. so that no agglutination of the staphylococci would occur. It should be noted that in the tube containing antistaphylococcus serum there was no apparent inhibition of growth of the staphylococci as compared with that in the control tube. In other words, although the liquefaction of gelatin was completely inhibited, the immune serum exerted no apparent antagonistic action towards those metabolic activities of the staphylococcus which are essential to its growth and multiplication.

TABLE XI.

Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.

Gelatin 4 cc. +		Liquefaction of gelatin after incubation at 20°C.		
Culture 0.000001 cc.	Serum 1 cc.	3 days.	6 days.	9 days.
Staphylococcus pyogenes aureus.	Antistaphylococcus serum.	−	−	−
" " "	Normal rabbit serum.	++	++	++

Experiment 10. Inhibition of Pigment Formation by Antistaphylococcus Serum.—When staphylococci were grown on the surface of serum agar slants and in serum agar shake cultures, it was found that antistaphylococcus serum exerted no inhibitory effect on pigment formation by the organisms. It was felt that under these conditions the serum might not be in sufficient concentration in the immediate vicinity of the staphylococcus colonies to exhibit any demonstrable antiblastic action. In order to obviate this objection as far as possible, serum agar plates were prepared (serum 1 part to plain agar 4 parts) and a thin layer of serum was spread over the surface of the agar which was then streaked with a loop of

staphylococcus culture. The plates were sealed to prevent evaporation of the serum as far as possible and incubated at room temperature. Under these conditions the growing staphylococcus colonies were in intimate contact with the serum. It was found that antistaphylococcus serum definitely retarded the formation of pigment by *Staphylococcus pyogenes aureus* (Table XII), although it exerted no apparent inhibitory action on the rate of growth of the bacteria, as compared with the growth in the presence of normal serum.

TABLE XII.

Inhibition of Pigment Formation by Antistaphylococcus Serum.

Materials used.	Pigment formation.		
	After 24 hrs.	After 36 hrs.	After 48 hrs.
Antistaphylococcus serum agar + *Staphylococcus pyogenes aureus*	−	−	±
Normal serum agar + *Staphylococcus pyogenes aureus*	±	+	++

This inhibition of pigment formation by antistaphylococcus serum must be attributed to some definite antagonistic property of the serum toward this function of the staphylococcus. It should not be concluded, however, from this inhibitory phenomenon that the antagonistic action of the serum is definitely antienzymotic in nature, for it is not definitely known that pigment formation by *Staphylococcus pyogenes aureus* is caused by enzyme action. Certainly the formation of pigment is not essential to the life and growth of the staphylococcus, for it does not occur during the early stages of growth and appears only after the staphylococcus colonies are well developed and multiplication probably has in large measure ceased. Further experiments are necessary to establish the exact nature of this inhibitory action of antistaphylococcus serum.

Experiment 11. Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.—To determine the inhibitory effect of antistaphylococcus serum on the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus*, two series of experiments were made, one with the use of a 5 per cent suspension of washed rabbit corpuscles, the other with a solution of hemoglobin prepared from rabbit corpuscles as in the pneumococcus experiments. A typical experiment is shown in Table XIII.

TABLE XIII.

Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.

Suspension of rabbit corpuscles 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
Staphylococcus pyogenes aureus.	Antistaphylococcus serum.	±	++
" " "	Normal rabbit "	++	++
—	" " "	−	−

Hemoglobin solution 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
Staphylococcus pyogenes aureus.	Antistaphylococcus serum.	−	±
" " "	Normal rabbit "	++	++
—	" " "	−	−

It was found that the reduction of oxyhemoglobin was not inhibited by antistaphylococcus serum when rabbit corpuscles were used, but that inhibition occurred when a hemoglobin solution was substituted for the corpuscle suspension. This difference is readily explained on the basis of the results obtained in the pneumococcus experiments. In the tubes containing rabbit corpuscles the blood cells rapidly settled to the bottom of the tube where they were in intimate contact with the agglutinated staphylococci, and were therefore in a position to be acted upon by the organisms. In the tubes containing hemoglobin solution, however, the agglutinated staphylococci were not in intimate contact with the hemoglobin held in solution throughout the medium and consequently were unable to act upon it within the time limits of the experiment. When the agglutinated clumps of staphylococci were broken up and dispersed throughout the medium by shaking, the hemoglobin solution was rapidly reduced. From these experiments it is evident that antistaphylococcus serum possesses no specific antiblastic property which enables it to inhibit the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus.*

DISCUSSION.

The series of experiments reported in this paper were undertaken in an attempt to explain the mechanism by which immune serum is able to retard or inhibit the metabolic activities of bacteria. This property of immune serum has been attributed by previous investigators to an antienzymotic or antiblastic action of the serum which exerts itself at the point of contact of the bacterial cell with its environment. While it has been clearly demonstrated in confirmation of the work of other observers that under certain experimental conditions immune serum does exert an apparent inhibitory action on the nutritional and certain other metabolic processes of bacteria, the facts brought out as the experiments progressed have made it seem probable that the inhibitory action exerted by antipneumococcus serum is not due to a specific antienzymotic or antiblastic property of the serum, but rather to its agglutinating properties. It is well recognized that many bacteria grow readily in homologous immune serum, provided the serum does not possess definite bactericidal properties, a fact which in itself would tend to throw some doubt on the validity of the antiblastic theory as applicable to all immune sera, since metabolic activity is considered to be essential to bacterial nutrition and growth. Bacteria when cultivated in media containing immune serum grow in agglutinated clumps at the bottom of the culture tube. Under these conditions they do not come into intimate contact with the whole medium and are unable to bring about changes rapidly throughout the medium. Presumably the metabolic activities which bacteria carry on in the process of growth exert their influence on the medium only in the immediate vicinity of the agglutinated organisms during the early stages of growth, and only as gradual diffusion of the medium takes place are the bacteria able to act upon the whole medium in sufficient degree to bring about those changes which indicate that bacterial metabolism is taking place. That these considerations hold with respect to the influence exerted by antipneumococcus serum on the metabolic activities of the pneumococcus has been demonstrated in the experiments reported above, in which it has been shown that the metabolic activities of the pneumococcus do take place in the immediate vicinity of the agglutinated bacteria in spite of the

presence of immune serum; that the degree of apparent inhibition which antipneumococcus serum exerts upon the metabolic activities of the pneumococcus depends upon the extent of agglutination of the organisms; that antipneumococcus serum exhausted of its agglutinin content possesses no antiblastic properties; that antipneumococcus serum possesses the property of apparently inhibiting the metabolic activities of entirely unrelated bacteria provided those bacteria grow in sedimented clumps in the presence of the serum; and that when pneumococci are grown in the presence of antipneumococcus serum under conditions that enable them to come into intimate contact with the whole medium, no inhibition of metabolic activity takes place.

A limited number of experiments with antistaphylococcus serum has confirmed the results obtained with antipneumococcus serum in as far as the inhibitory effect of the serum on the reduction of oxyhemoglobin by the staphylococcus is concerned. On the other hand, antistaphylococcus serum has been found to inhibit or markedly retard the formation of pigment and the liquefaction of gelatin by *Staphylococcus pyogenes aureus*. Under the conditions of the experiments agglutination of the bacteria did not occur and therefore could not have been a factor in causing the inhibition. It is to be noted that neither pigment formation nor the liquefaction of gelatin is an essential process in the nutrition of the staphylococcus and that these phenomena take place in demonstrable amount only after the staphylococcus colonies have become well developed. It is felt that further experimental work is necessary to determine the exact mechanism of the inhibitory action of antistaphylococcus serum on these functions of the staphylococcus before the conclusion can be safely drawn that it is of an antienzymotic nature. The outstanding fact that staphylococci as well as pneumococci grow rapidly and abundantly in the homologous immune serum is sufficient in itself to indicate that the immune serum possesses no properties in demonstrable amount antagonistic to those bacterial metabolic activities essential to the growth and multiplication of the bacteria.

CONCLUSIONS.

Antipneumococcus serum under certain conditions apparently inhibits or retards the metabolic activities of the homologous pneumococcus.

Antipneumococcus serum exhausted of its agglutinin content possesses no inhibitory properties.

The degree of inhibitory action of antipneumococcus serum parallels its agglutinating power.

No evidence has been found to indicate that the inhibition of the metabolic activities of the pneumococcus by antipneumococcus serum is due to a specific antienzymotic property of the serum.

The evidence which has been obtained indicates that the apparent inhibition of metabolic activity that occurs under certain conditions is due to agglutination of the pneumococci by the antipneumococcus serum and their consequent inability to grow in intimate contact with the whole medium.

Antistaphylococcus serum inhibits the liquefaction of gelatin and the formation of pigment by *Staphylococcus pyogenes aureus*. Further experimentation is necessary to determine the mechanism of this inhibitory action.

Antistaphylococcus serum does not inhibit those metabolic activities of *Staphylococcus pyogenes aureus* essential to the growth and multiplication of the organisms.

FURTHER STUDIES IN EXPERIMENTAL ATHEROSCLEROSIS.

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PLATES 40 TO 43.

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Several years ago, the writer[1] reported some experiments on dogs as a contribution to the study of experimental atherosclerosis. Since that preliminary report, the experiments have been continued and though much of these very elusive problems is still far from being clear, it is thought best to report some of the results of further study. It may be well to summarize briefly the contents of the article just referred to. Infectious disease has frequently been insisted upon as an important etiological factor in atherosclerosis. In order to test this, dogs were infected with pure cultures of various pathogenic bacteria and also with bacterial toxins, by means of injections into the jugular vein. The animals were kept in a state of mild chronic sepsis for periods ranging from 4 to 7 months. The results, as far as the blood vessels were concerned, were entirely negative. Lead and nicotine also gave negative results. General overexertion maintained for long periods by means of a treadmill, both with and without the hypertension produced by the injection of large doses of adrenalin, also proved without effect. The parenteral methods were then abandoned and feeding experiments were taken up. Some attempts were made with cottonseed-oil, which, however, proved too toxic. Then two dogs were fed daily with pure cholesterol in half-gram doses enclosed in capsules. These two dogs died after 1½ and 2½ months respectively. In the former publication positive results are claimed for these two experiments, because a number of whitish yellowish streaks, very slightly raised above the level of the intima, was found in the aortas. In the light of later experience these can no longer be considered as positive results. The doses of cholesterol were too small, dogs eliminate cholesterol more rapidly and completely than rabbits, and under these conditions 1½ and 2½ months would appear altogether too short a time for the development of so insidious and chronic a lesion as atherosclerosis. Moreover, experience has shown that these insignificant ridges and patches are found fairly regularly in the aorta of young

[1] Adler, I., Studies in experimental atherosclerosis. A preliminary report, *J. Exp. Med.*, 1914, xx, 93.

and healthy individuals both canine and human. There will be occasion to speak of this more in detail presently. It is not necessary to recount here all the varieties of experimentation that were attempted. It is important however to mention two dogs that received, besides their usual food of bread and meat, dilute hydrochloric acid up to sixty drops daily for 2 months and 3 months respectively. Both these dogs showed very definite and unmistakable arteriosclerotic patches in various portions of the aorta and in the one case, especially well marked in the lowest portion of the abdominal aorta. There were no traces of ulceration or calcification. Microscopically these patches showed the typical picture of arteriosclerosis, the details of which need not be gone into, except to mention the fact that cholesterol, in its free state, was abundantly present. That these findings could not be accepted unhesitatingly as proving hydrochloric acid to be an important factor in the etiology of arteriosclerosis is obvious. Two experiments are altogether insufficient to clinch so important a conclusion. The time during which the hydrochloric acid feeding was continued was too short, and more important than everything else, the age of the animals could not be ascertained and both dogs were in all probability rather old. It is easily possible, therefore, that the lesions found were not due to experimentation but were spontaneous, for it can no longer be doubted, though it is perhaps not generally recognized, that arteriosclerosis, closely analogous to the human, occurs spontaneously in old and debilitated dogs. It was necessary therefore to continue these acid experiments on a little broader basis.

The writer is still of the opinion that if it be possible to produce atherosclerosis, analogous to, or identical with the human type, experimentally and with reasonable certainty, the methods employed should give positive results not only in rabbits, but with a fair constancy in all animals that are subject to spontaneous or typical senile atherosclerosis. Unfortunately animal pathology has been more or less neglected in this respect, and it is possible that when more is known of vascular disease in the animal kingdom, more favorable subjects for the experimental study of atherosclerosis will be found. Until then, however, dogs would seem sufficiently well adapted for this class of experiments. They are omnivorous as the human; the structure of their blood vessels, especially of the aorta, differs not so very greatly from the structure of human arteries; and while they are subject to pronounced senile atherosclerosis, the lesions are not obscured and rendered uncertain by such forms of median necrosis as occur spontaneously in so large a percentage of rabbits. It is the writer's firm conviction, therefore, that the following conditions

should be fulfilled before it can be justly claimed that a genuine atherosclerosis has been artificially produced by experiment. (1) The gross lesions must be closely analogous, if not quite identical in every microscopic detail with the human arteriosclerosis. (2) These positive results must be so constant and regular that it is reasonably to be expected that every experiment will give a positive result, with perhaps only an occasional failure under exceptional conditions. Definite conclusions from apparently positive findings in only one or two animals are not permissible. The number of positive results must be sufficiently large, though an exception here and there may occur. In man also there occur instances, from the celebrated case of Harvey's down to modern times, where persons can live even to extreme old age, apparently immune to arteriosclerosis, their arteries remaining soft and elastic up to their death. (3) In order to avoid the probability of the lesions being due to spontaneous degeneration, the experiments should all be done on young animals. It might be objected to this that the youthful organism is perhaps possessed of powers of resistance and repair which are materially diminished or lost in older individuals and that therefore experiments on very young animals would not be likely to give positive results by the method in question even if they fulfilled all conditions on older individuals. In answer to this it may be said that since it has of late years been positively demonstrated that very young human individuals, even babies, are subject to unmistakable arteriosclerotic lesions under certain conditions, it seems more than probable that the same is true of dogs.

It was now necessary to test the action of acids to better advantage. The acids employed were: (1) hydrochloric acid, to verify if possible the apparently positive result that had been obtained in the two former experiments; (2) lactic acid, because O. Loeb[2] had claimed positive results with this acid and had developed a plausible theory concerning the mechanism of its action; (3) acetic acid, selected because of its occasional occurrence in certain types of digestive disturbance. Other acids, as for instance butyric, might logi-

[2] Loeb, O., Ueber Arteriosklerose. II. Ueber experimentelle Arterienveränderungen mit besonderer Berücksichtigung der Wirkung der Milchsäure auf Grund eigener Versuche, *Deutsch. med. Woch.*, 1913, xxxix, 1819.

cally and profitably have also been tested, but for various reasons this could not very conveniently be done.

Hydrochloric Acid.

The series includes three dogs. All of them are young. Two were males, one female. In every case the ordinary chemically pure hydrochloric acid (specific weight not determined) was used. The initial dose was ten drops mixed with the food of the animal and increased by five drops every 3rd day. In every instance it took some time until the animals became accustomed to this unusual admixture to their diet. They would leave portions of their food, and at times would not eat at all; sometimes the dose of acid would have to be diminished; at other times the administration of the acid would have to be stopped altogether for a few days. But after a longer or shorter period the animals became thoroughly habituated to the acid and ate with great appetite, while the doses of acid were steadily increased. This is true, it should be said, not only of the hydrochloric acid series but of the two other acids as well. In the hydrochloric set there was one experiment (No. 1) that was disappointing.

Experiment 1.—Brown dog, male; weight 8,500 gm. Dec. 5, 1914. Commenced with 10 drops increased by 5 drops every 3rd day. Eats well until Dec. 28 by which time the dose reached 65 drops. Now begins to leave some food and to lose weight. Dec. 30. Weight, 7,600 gm. After a few days of 70 drops, dog is kept at 60 from Jan. 1, 1915, to Feb. 10. During all this time, eats well but loses weight steadily. 70 drops up to Feb. 16. Refuses food entirely for 2 days. Feb. 18. Died. Weight at death 5,500 gm. Experiment had lasted only about 2½ months during which time the dog took about 250 cc. of hydrochloric acid.

Besides the usual small punched out erosions in the stomach and the upper intestine and a great number of round worms, there are no definite lesions apparent, except general atrophy and marasmus.

Experiment 2.—Young brindle bull, female; weight 9,500 gm. Dec. 1, 1914. Commenced with 10 drops as usual. After a few days, weight begins to decrease. Acid has to be stopped, then again resumed; sometimes the dog eats well, at other times leaves great portions of food. Weight increases slightly, then is again diminished, the lowest weight being 6,800 gm. on Mar. 22, 1915. Appetite, however, is excellent and 80 drops of acid are given daily. From now on the dose of acid is increased by 10 drops about every 2 weeks. Weight increases steadily;

the animal is in excellent condition. Aug. 25. The dog gives birth to six healthy pups. From July 6 to Oct. 19, when the animal was killed, 140 drops were taken daily. Duration of the experiment 10 months, 19 days; total amount of hydrochloric acid consumed somewhat over 2,000 cc.

The autopsy shows a very slight hyperemia in scattered areas through the gastrointestinal tract. Otherwise there is nothing abnormal.

Experiment 3.—Black and white dog, male; weight 8,700 gm. Feb. 19, 1915. Dog started at once at 40 drops but though it does not eat well and is losing weight, the dose is steadily increased; weight varies. Though there are times when it absolutely refuses to eat, the dose is still increased and the dog begins to gain steadily in weight. Oct. 15. Killed; weight 9,700 gm. From July 30 to Oct. 15 the dog had taken 140 drops daily, ate all the food, and seemed in excellent health. The experiment lasted about 8 months, during which time the dog had consumed a little more than 1,600 cc.

The autopsy shows besides the usual intestinal parasites, fairly normal conditions. There is not even any very extensive hyperemia in the stomach and intestines.

Lactic Acid.

On the whole it seems that the animals did not take to lactic acid quite so easily as they did to hydrochloric.

Experiment 4.—Young fox-terrier, female; weight 6,000 gm. Dec. 1, 1914. Commenced with 10 drops daily; after a week 20 drops were given, and animal refused food, and weight decreased very rapidly. Dec. 10. The lactic acid is stopped altogether. Feb. 16, 1915. Weight is about 4,800 gm. The animal is kept on normal food without any acid until June 4. On that day having been eating well and being in good condition, lactic acid is given again with an initial dose of 90 drops. From now on appetite is excellent and increase in weight steady. The dosage of lactic acid is rapidly increased and from Sept. 19 until Oct. 14, when the dog is killed, 140 drops are taken daily. Weight at death 11,606 gm. The dog was under observation for 10½ months, but the lactic acid experiment, counting from June 4, 1915, lasted a little over 4 months in which somewhat more than 1,000 cc. were administered.

The autopsy shows some patchy hyperemia in the gastrointestinal tract, otherwise no organic lesions. No definite macroscopic signs of arteriosclerosis, although the yellowish ridges and patches in the arch and in the descending aorta are a little more pronounced than usual.

Experiment 5.—Bull-terrier, male; weight 13,000 gm. Dec. 1, 1915. Similar history. Eats well for several weeks, though weight decreases. Leaves large portion of food for about a week then begins to eat well again. Weight and ppetite vary. Sometimes the dose of lactic acid had to be diminished; some ames stopped altogether for a day or two. The dose had been increased to ti

175 drops, then it had to be diminished again to 80. Feb. 24, 1916. Lowest weight reached 10,300 gm. From now on dog increases steadily in weight and eats everything. Oct. 8. Killed. From June 14 to Oct. 8, 200 drops of lactic acid taken daily. The experiment lasted about 10 months, the total amount of lactic acid being approximately 3,000 cc.

At the autopsy no gross lesions of any kind are found.

Experiment 6.—Brown and white cur, female; weight 10,700 gm. Dec. 29, 1914. History similar to the foregoing. It took until about Feb. 24, 1915, for the animal to become accustomed to the lactic acid. On that day the minimum weight of 6,500 gm. was reached, but from now on the dog showed excellent appetite, ate everything it could get, steadily increasing in weight. Oct. 13. Killed. From June 4 to Oct. 13, 160 drops were taken daily, and the weight at death was 12,700 gm. The experiment lasted about 9½ months. Total amount of lactic acid consumed somewhat over 2,000 cc. The dog had given birth to a litter of pups 2 days before death.

The autopsy shows no gross lesions.

Acetic Acid.

Again the history is about the same as in the foregoing two series. 50 per cent glacial acetic acid, chemically pure, was used.

Experiment 7.—Brindle bull, male; weight 8,100 gm. Dec. 29, 1914. There are the usual variations in weight up to a certain lower limit. The dosage of acid is increased or diminished according to the amount of food the animal takes. It takes till about Mar. 9, 1915, for the dog to become fully accustomed to the acid. From now on its appetite is excellent, health good, and weight steadily increasing. Oct. 7. Killed. From June 14 to Oct. 7 the daily dose was 150 drops. Weight on day of death 12,000 gm. Experiment lasted 9 months and 10 days. Total quantity of glacial acetic acid consumed approximately 2,000 cc.

The autopsy shows no gross lesions.

Experiment 8.—Coach dog, male; weight 9,700 gm. Dec. 1, 1914. There are similar variations in weight and in dosage. It takes till Feb. 23, 1915, for the dog to become accustomed to the acid. From then on, dose of acid is steadily increased, and the weight increases though there are occasional bloody stools. June 1. Killed in a fight. Weight at death 10,200 gm. From Apr. 25 to date of death 150 drops of acid were taken daily with excellent appetite. Experiment lasted 6 months. Total amount of glacial acetic acid approximately somewhat over 1,000 cc.

The autopsy shows no gross lesions.

It is not necessary to give any details of the microscopic examinations. No lesion was discovered which could be interpreted as anal-

ogous to arteriosclerosis. The positive findings in the two dogs reported by the writer several years ago were, therefore, evidently not due to the hydrochloric acid, but were spontaneous and probably of the senile type. Loeb's claims of positive results with lactic acid are also not convincing. The one dog 8 years old, Loeb himself disregards on account of his age. In the other dog, only 1½ years old, he regards as arteriosclerotic various small ridges and patches in the intima of the aorta. He gives no microscopic details and it is therefore not at all unlikely that these patches and ridges were no more significant than those found so frequently in the aorta of normal dogs, both young and old. It can therefore be safely asserted that the feeding of dogs with large quantities of various acids for a comparatively long period, though the cause of weeks of severe indigestion and marasmus, has not resulted in any lesions comparable to arteriosclerosis.

As cholesterol still appears to occupy the most prominent place in studies on arteriosclerosis, it was resolved to revert once more to experiments with that substance. Meanwhile a paper by Klotz[3] had appeared reporting the effects of injections into the ear vein of rabbits of cholesterol dissolved in pure oils or as a soap emulsion. It was decided therefore to try somewhat similar experiments on dogs.

Some attempts were made to develop a technique by means of which cholesterol was to be injected directly into the heart or into the aorta. The only result of these tentative experiments was the production of some cases of hemorrhagic and exudative pericarditis. It was then decided to inject cholesterol through the jugular veins. This series includes four dogs. All four were young animals, probably barely a year old. The cholesterol was obtained from gall stones and thoroughly purified; it was dissolved in sesame oil which was cheap and practically non-toxic. Its phytosterol content, being as in all similar oils less than 1 per cent, could, in the small quantities used in each injection, be considered negligible. It was found that 0.2 cholesterol in about 8 cc. of sesame oil at body temperature gave a perfectly clear solution. This solution was employed exclu-

[3] Klotz, O., Vascular changes following intravenous injections of fat and cholesterin, *J. Med. Research*, 1915–16, xxxiii, 157.

sively. Any attempt to increase the quantity of oil or use a higher concentration of cholesterol gave rise to more or less grave disturbances, as for instance, acute pulmonary edema and rapid death of the animal. For the first few injections, ether was given and a very small incision into the skin was made and the jugular laid bare. It soon became evident, however, that the incision was not necessary and that the vein could be reached with certainty, after a little practice, through the intact skin. Two injections a week were given regularly and without any exception for a period of between 8 and 9 months. The total amount of cholesterol consumed by each animal during that period was about 11 gm. No deleterious effect of the injection was noticed at any time. To observe whether a permanent rise in blood pressure had any effect upon the result of the injections, in two of the dogs, before any injections were made, the abdominal aorta was tied by the Halsted[4] method. A flap of the fascia lata was taken, with which the aorta was tied somewhat below the right renal artery. The ligature was tightened so that pulsation in the femoral artery could just be felt. In both cases the operations were uneventful and the wounds healed without any difficulty.[5] As the autopsies later on showed, permanent stricture of the aorta was accomplished by this operation. This must necessarily have affected not only the pressure in the aortic system, but also, indirectly at least, in the pulmonary circulation into which the cholesterol was primarily discharged.

The further history of these four dogs is uneventful. During the very hot and humid summer of 1916, the animals suffered from the mange, but with the coming of cool weather and with the necessary dermatological attention, they recovered fairly well, and were in good condition when they were killed. One dog did not recover its health entirely after the mange was cured, but continued losing weight. The injections were, however, continued regularly. On November 16 the dog was killed. At the autopsy it was found that the entire intestinal tract, including stomach and colon, showed hemorrhagic

[4] Halsted, W. S., Partial occlusions of the thoracic and abdominal aortas by bands of fresh aorta and of fascia lata, *Proc. Soc. Exp. Biol. and Med.*, 1912–13, x, 113.

[5] The operations were performed by Dr. M. J. Sittenfield, to whom I am indebted for his valuable assistance.

swellings with partial destruction of the muscularis. The contents of the intestines were bloody and the mucosa itself was in many places hemorrhagic. There were numerous ulcers, some of them extending down to the serosa. Great numbers of *Ascaris mystax* were present. In all four dogs the lungs at autopsy were found much firmer than normal and not completely collapsed. There were also present small wedge-shaped hemorrhagic infarctions, and patches of brown pigmentation evidently due to old hemorrhages. The liver, kidneys, and pancreas appeared normal. The adrenals were not enlarged and showed no gross lesions. The spleen in nearly every case was somewhat enlarged and showed very large prominent follicles. In one case there was some softening of the pulp, probably due to hemorrhage. In one of the dogs, in which the aorta had been tied, a definite, fairly close stricture of the vessel was seen surrounded by an area of thickening, but there was no noticeable dilatation proximal to the obstruction. In the other operated dog, there was also an area of thickening about the place of ligature, but the aorta appeared of practically uniform caliber, possibly very slightly constricted at the place where the ligature had been applied. No gross lesion could be recognized in any of the jugulars and the microscopic examination also showed perfectly normal conditions, with only here and there a small droplet of fat in the adventitia. Neither the frequent passage of oil and cholesterol through the vein nor its puncture several hundred times by a rather large needle seems to have had an injurious effect upon the delicate vessel. No trace of inflammation or degeneration could be seen. The heart muscle in every instance was flabby and was found in diastolic dilatation. There was no hypertrophy of the right ventricle. The animals were killed by the injection of chloroform into the jugular vein, and to this the flabby and dilated condition of the heart muscle may be ascribed. The aortas showed some ridges and patches and wrinklings here and there, more frequently around the orifices of branching vessels. These irregularities in the intima of the aorta are usually scarcely visible to the naked eye, especially in the fresh object, unless one has become familiar with them and searches for them carefully. After the artery has been kept in formalin for some time, they become a little more distinct. Besides these, however, more extensive sclerotic thickenings were plainly visible. This was

especially the case in one aorta (Fig. 1) in which, in the sinus Valsalvæ behind the semilunar valves, these sclerotic areas protruded more distinctly. In the bulb of this aorta also and near the orifices of the branching intercostal arteries a number of ridges was noticed which tended to confirm the impression of incipient sclerosis.

The oil and cholesterol injected into the jugular is of course carried through the upper cava and the right auricle into the right ventricle, and from there through the pulmonary artery into the lungs. It might be confidently expected, therefore, that if any changes in the arteries are to result from this procedure, they would show primarily in the pulmonalis, rather than in the aorta, for the first powerful impact of the blood laden with cholesterol is directed against the former. This expectation was completely verified. In all four dogs very definite changes, producing the distinct impression of arteriosclerotic thickening and loss of elasticity, appeared in the pulmonalis (Fig. 2). There were no signs of ulceration or calcification nor was there any appreciable dilatation. These departures from the normal were principally of the nodular type, protruding more or less prominently into the lumen of the vessel. They commenced immediately behind the valvular cusps, showed the faint whitish yellowish tint common to all sclerotic lesions of the intima, and extended, more or less, further up into the vessel. These nodulations were most pronounced in the two dogs in which the aorta had been tied. In the other two dogs the lesions, though quite definite and plain, were much less in extent and prominence. It is not possible to say whether this is merely coincidental, or whether the increased blood pressure is responsible. The most marked sclerotic changes were found in the dog whose aorta showed comparatively slight obstruction. While the gross lesions of the main trunk of the pulmonary artery closely resembled ordinary intimal arteriosclerosis before the development of atheroma has begun, the microscopic examination revealed a condition entirely unexpected, and in many ways rather surprising. Hypertrophy of the intima with proliferation of the endothelium, new formation of connective tissue, and the splitting of the elastica interna as described by Jores was distinctly in evidence. This hypertrophy of the intima however was comparatively limited and not commensurate with the amount of thickening. In many places there was hardly any prolif-

eration of the intima or it was entirely absent. The most pronounced and constant and evidently primary lesions were found in the media, and especially in its innermost portions adjoining the elastica interna. Within this region there had taken place much damage to the elastic tissue. Everywhere the breaking up of elastic fibers into larger and smaller pieces and into granules (*Körnchenzerfall*) could be seen. Very often it appeared as if the elastic fibers, especially the larger ones, had suddenly snapped and the ends could be seen some distance apart coiled like the lash of a whip. Another lesion, evidently secondary to this forcible disruption of the elastic tissue, was the separation of the muscular elements into bundles of varying dimensions and at varying angles, which were so pushed aside as to leave wider or narrower gaps between them. There seems to be no doubt that this disorganization of the elements of the media was largely responsible for the prominent areas of apparent thickening, and that the proliferation of the intima took only a minor and secondary part in the process (Fig. 3). These comparatively wide gaps between the dislocated groups of muscle cells usually contained remnants of broken up elastic fibers, but otherwise appeared empty (Fig. 4). That they were not empty could be surmised from the compactness and solidity of the nodules. Edema could be positively excluded; staining for fibrin or mucin gave no results, but with certain stains, as for example alum-carmine, the faintest possible tinge could be obtained, sometimes showing slight inequalities and suggesting some very delicate coagulated material. It is probable that these wide areas contain, as Ribbert[6] suggested, coagulated blood plasma. He finds these gaps, however, only in the intima, where he believes they are the effect of excessively high blood pressure. He attributes the deposit of cholesterol to this also. No sign of necrosis could be detected and there was nothing to suggest the median necrosis as seen in rabbits or in human arteries. The muscle cells, although detached from their normal position were in perfect condition, and the elastic fibers though shattered and disorganized retained their normal staining properties. There were no inflammatory

[6] Ribbert, H., Über die Genese der arteriosklerotischen Veränderungen der Intima, *Verhandl. deutsch. path. Ges.*, 1905, viii, 168.

symptoms and the vasa vasorum were normal. It seemed clear that the entire process was purely mechanical. The sclerotic areas in the aorta showed the same median disorganization even more strictly confined to the media, for the intima was mostly not affected, but normal.

The lungs presented a very constant and characteristic microscopic picture. Even a superficial examination showed that the bulk of the oil and its cholesterol content was filtered out through these organs. The sudanophil masses were deposited almost exclusively in the interalveolar septi, and there mainly in the capillaries. The latter were much distended and filled to bursting with oil. Very frequently the distended capillaries were surrounded by an infiltration of leukocytes, both processes together causing a considerable thickening of the septi with very marked encroachment upon the alveolar air spaces. The alveoli themselves were practically free from oil. Here and there an overdistended capillary would burst and a few drops of oil be left in the alveoli. Dispersed throughout the lung there were clear indications of hemorrhages. Numerous red blood corpuscles were found in the air spaces and in the septi, and hemorrhagic infarctions were also irregularly scattered throughout the lungs, especially near the margins. Notwithstanding all this the pulmonary apparatus seemed quite adequate to combat the enormous overburdening caused by the oil injections. Clinically there were no symptoms of respiratory disturbance and this could be accounted for by the fact that though the pulmonary circulation was heavily overtaxed, nevertheless capillaries and alveoli always remained unaffected in sufficient numbers to ensure the necessary aeration of the blood. There is, moreover, good reason to believe that the oil was very rapidly discharged from the lung. If the dog was killed within 24 hours of an oil injection the lungs were found overcrowded with sudanophil masses, while if 2 or 3 days had elapsed between the injection and the killing, comparatively little sudanophil material remained, and the lungs presented an appearance more nearly approaching the normal. It appears that there is a marked difference between the reaction to cholesterol in oil as shown in the lungs of the dog and that described by Klotz[3] in the lungs of the rabbit. A very careful study of a large number of sec-

tions of the lungs of all the dogs and from all parts of the lung failed to show any crystals or any doubly refracting substances. Phagocytes with granular fatty inclusions were scattered throughout the lungs, mostly in the septi, occasionally in the alveoli. Extensive areas of the lung however appeared free from phagocytes. On the other hand, it is the writer's impression that in certain localities, often near the margins, or where the pulmonary tissues had become more or less indurated by hemorrhagic infarctions and the subsequent reactive processes, and where accordingly the circulation was materially interfered with and the blood vessels were presumably no longer able to carry off the fatty material with sufficient dispatch, the phagocytes accumulated in considerable numbers, presumably to replace or rather materially to aid the work of the blood vessels. In such spots large phagocytic cells filled with granular sudanophil material are heaped in close proximity. Frequently several of these cells merge into one large giant cell, the several nuclei being distributed irregularly, some in the center and others pushed aside towards the circumference. Many large cells filled with granules of brown or blackish pigment and resembling very closely the *Herzfehlerzellen* are also found in these indurated areas.

The blood vessels in the dog's lung also showed somewhat different reactions from those described by Klotz for the rabbit. The pulmonary veins as well as the bronchial arteries appeared to be normal. The capillaries also, notwithstanding the tremendous strain to which they were subjected by the overcrowding with oil, did not show structural lesions. The smaller and smallest branches of the pulmonary artery were also without exception normal. It was only in the right and left main branches of the pulmonary trunk and their subdivisions of the second and third order that definite structural changes in the vascular walls were found. It was impossible to decide whether there was any dilatation of these larger vessels as there were no normal measurements available that might have served as a standard, but the general impression was that if any dilatation had actually taken place, it could not be very serious. Several small globular nodules were found in the two main branches, though they were not so large or so extensive as those described in the main trunk. The microscope showed that these little globules were the

result of a very marked and peculiar hyperplasia of the media. The elastic lamellæ and the muscle fibers were not arranged in the usual symmetrical layers but formed more or less regular concentric circles or in other places abutted upon each other at various angles, thus producing upon the whole the impression of a whorl of tissue. Within these peculiar nodulations some of the elastic fibers had also given way and snapped asunder and here also spaces apparently filled with plasma and containing remnants of broken elastic tissue occurred. The dislocation and forcing apart of the elements of the media, and the destruction of elastic tissue are, however, minimal when compared with the same processes as seen in the main trunk. Another peculiarity of this strange form of nodulation is, that it is practically impossible to decide where the media ends or the intima begins. No elastica interna is visible, and the innermost layers of the vessel consist of fibrous and muscular tissue all included in a fine network of delicate elastic fibers forming irregular meshes, and the whole is lined with moderately proliferating endothelium. Rather small phagocytes are occasionally, but on the whole rarely to be found, as also very minute sudanophil droplets between the cells. Very definite and typical hyperplasia of the intima, independent of those hypertrophic median nodules, is also found in the two main branches, but especially in some of the larger arteries well within the lungs (Fig. 5). Here there is comparatively little change in the condition of the media although very small spaces filled with plasma and containing fragments of elastic fibers are sometimes seen just below the elastica interna. These thickenings of the intima, though they distort the lumen of the vessel but slightly, agree accurately with the description given by Jores.[7] There is the splitting of the elastica interna, the new formation of fibrous and muscle cells, and some proliferation of the endothelium, but there is this difference, that there is no pronounced fat phanerosis or fatty degeneration. It is true that some fat phagocytes and minute fatty droplets are found in the intima and even sometimes in the inner layers of the media, but they are few in number and not constant in occurrence or regular in distribution.

[7] Jores, L., Wesen und Entwickelung der Arteriosklerose, Wiesbaden, 1903.

It is obvious, therefore, that though the pulmonary circulation was for many months overcrowded with cholesterol in oil; though there must have existed a certain amount of hypercholesteremia, as shown by the excess of sudanophil bodies appearing throughout the heart muscle, the liver, spleen, kidneys, and adrenals; and though the pressure within the pulmonary, and also, though perhaps to a lesser degree in the aortic system, must have been very great; the cholesterol evidently took no essential part in the processes that led to such profound changes in the structure of the arterial walls.

What relations if any, do these findings bear to human atherosclerosis? It is now very generally believed that the latter is an affection primarily of the intima, essentially degenerative in character, and that cholesterol and its esters are very important factors in this process. The results of the present experiments on the other hand are apparently in direct opposition to this theory. Though to the naked eye the nodules protruding into the lumen of the vessels, and also the occasional diffuse thickening of the vascular walls, closely resemble arteriosclerosis at a stage in which ulceration and calcification have not as yet begun, the real localization of the lesion is found to be mainly in the media. The structural disruptions and dislocations appear to be the result of the action of mechanical forces rather than of chemical degenerative reactions. The hypertrophy or hyperplasia of the intima is obviously merely secondary to the lesions in the media and lastly it would appear that cholesterol does not count as a factor in the process. Notwithstanding these seemingly important differences, the present writer is nevertheless of the opinion that a very close analogy, if not identity, exists between human atherosclerosis of the pulmonary blood vessels and the lesions produced experimentally in our dogs. Our knowledge of human atherosclerosis is as yet far from complete. The controversy between those who assume a mechanical disturbance as primarily the cause of arteriosclerosis, those on the other hand who are more inclined to make toxic and other chemical influences responsible, and those who, pursuing a middle course, seek to explain the sclerotic phenomena by combining in various ways the extreme views of both sides, is still unsettled. Aside from the problems of atherosclerosis in general, however, what is known of sclerosis of the pulmonary system must

be taken specially into account. It is not many years ago that it was supposed that sclerosis of the pulmonary artery, if it occurred at all, was extremely rare. Bichat[8] seeks to show that vessels carrying venous blood seldom if ever show traces of sclerosis and hence, according to him, the pulmonary artery, in which only venous blood circulates, is practically exempt. During the last 12 to 15 years the subject has been more intensely studied. A very complete review of the literature on this subject has been given by Posselt,[9] and more recently by Ljungdahl.[10]

It is fairly certain now that pulmonary sclerosis is found quite frequently if it is only carefully looked for. Genuine senile sclerosis of the pulmonary artery seems to be quite rare, but according to Ljungdahl, contrary to the view of many authors, it does occur, but mainly in association with special complications, pulmonary emphysema for instance, that tend to raise the pressure within the artery considerably. Fischer[11] found in several cases of advanced atherosclerosis of the aorta and peripheral arteries, that the pulmonary arteries were healthy, but in these cases the lungs were free from emphysema or other chronic lesions and there was no hypertrophy of the right heart. Posselt finds that sclerosis of the pulmonary artery seems to affect youthful persons more readily than is the rule in sclerosis of the aortic vessels. According to this author 97 out of 187 cases were found in individuals under 40, 125 in subjects under 50, and only 60 in persons above 50 years of age. All authors, however, seem agreed that hypertension within the pulmonary circulation is a main factor in the etiology of sclerosis of the pulmonary artery, which is generally looked upon as an *Abnutzungskrankheit* as defined by Marchand.[12] The fact frequently observed that the artery remains intact, though the blood pressure is steadily much above normal, strongly suggests that hypertension is not the only factor, but that other etiological elements, such as local or functional variations, toxic or other influences as yet unknown, are also involved. For the pulmonary artery too, the dogma that the intima is the primary seat of the pathological changes is very generally accepted.

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[8] Bichat, quoted by Brüning, H., Untersuchungen über das Vorkommen der Angiosklerose im Lungenkreislauf, *Beitr. path. Anat. u. allg. Path.*, 1901, xxx, 457.

[9] Posselt, A., Die Erkrankungen der Lungenschlagader, *Ergebn. allg. Path. u. path. Anat., Ite Abt.*, 1909, xiii, 298.

[10] Ljungdahl, M., Untersuchungen über die Arteriosklerose des kleinen Kreislaufs, Wiesbaden, 1915.

[11] Fischer, W., Über die Sklerose der Lungenarterien und ihre Entstehung, *Deutsch. Arch. klin. Med.*, 1909, xcvii, 230.

[12] Marchand, *Verhandl. Kong. inn. Med.*, 1904, xxi, 23.

There are, however, some dissenting voices. Ehlers[13] finds that the human pulmonary artery differs from the aorta in that its media is made up of two distinct layers and according to him the innermost layer assumes a very active part in pathological processes. Durante[14] reports a case of congenital atheroma in a premature infant. He finds the vasa vasorum normal, the intima almost normal. The main process is in the inner third of the media where there is some necrosis and calcification. He also describes broad, almost unstainable gaps in the media. There is some secondary hypertrophy of the intima but only over points of most advanced degeneration of the media. The aorta appears macroscopically normal, but under the microscope it is seen that in its media there are similar processes developing as in the pulmonary. Except for the median necrosis and calcification, the conditions in this case seem to bear a striking resemblance to those obtained experimentally in our dogs.

These few brief notes from the literature will suffice to show that the results of the experiments here described present some obvious analogies to the sclerosis of the human pulmonary artery, but at the same time distinct differences. The proliferation of the intima wherever it is found is the exact counterpart of what is considered typical for human atherosclerosis, and in the section taken from a branch within the lung in which the media is barely affected (Fig. 5) the picture is identical with any that can be taken from an arteriosclerotic artery that has not as yet undergone an appreciable amount of degeneration. One of the chief points of difference is that in our dogs it is not the intima but the media which is without doubt the primary and principal localization of the lesion. That this is not necessarily peculiar to the dog but can be found also in human pulmonary arteries is sufficiently proved by the above quotations from Ehlers and Durante. Perhaps greater experience and improved methods may bring forward a greater number of similar human cases. Our experiments, moreover, have shown the process at a much earlier stage than is usually seen in the human. That the peculiar structure of the pulmonary artery as described by Ehlers is not to be held responsible for this localization in the media is obvious because identical lesions may be found at the same time in the aorta.

[13] Ehlers, W. E., Zur Histologie der Arteriosklerose der Pulmonalarterie, *Virchows Arch. path. Anat.*, 1904, clxxviii, 427.

[14] Durante, G., Athérome congénital de l'aorte et de l'artère pulmonaire, *Bull. Soc. anat. Paris*, 1899, lxxiv, 97.

One is apt, therefore, to recur to the original theory of Thoma[15] who assumed some defect in structure or in function of the media as the initial stage in the development of atherosclerosis. The almost uncomplicated hyperplasia of the intima as seen in some of the larger arteries within the lung itself might also be readily accounted for by the early theories of Thoma. These arteries are without doubt dilating under pressure, the current is probably considerably slower than normal, and hence the compensatory thickening of the intima. It might be assumed that the effect upon the media was comparatively slight because in arteries of that order the muscular apparatus is not sufficiently developed to exert any great resistance to the pressure within the artery. The disorganization in varying degree of elastic lamellæ and fibers is admitted by all observers but is generally conceived as taking place principally in the intima, and only to a very limited extent in the media. It is taken to be a direct consequence of fatty degeneration. According to Torhorst[16] degeneration does not commence before the completion of the hyperplastic process in the intima, and he as well as most other investigators, especially Aschoff,[17] see the cause of the breaking up of the elastic elements in the fatty degeneration of the cement (*Kittsubstanz*) that serves to hold the fibers together.

In the present set of experiments, no trace of degeneration, fatty or otherwise, could be found. The violent destruction of the elastic elements, the forcible displacement of the muscle cells, the broad gaps filled with plasma, all point to a purely mechanical force, in all probability the intense straining of the musculature of the arteries to overcome the resistance offered by the extensive blocking of the pulmonary circulation in the lung. It will be of great interest to observe whether the cholesterol injections, possibly with some modifications, and continued during a much longer period, will eventually lead to fatty degeneration and calcification, though Ljungdahl seems to believe that 7 to 8 months of increased resistance on the part of

[15] Thoma, R., Über die Histomechanik des Gefässsystems und die Pathogenese der Angiosklerose, *Virchows Arch. path. Anat.*, 1911, cciv, 1.

[16] Torhorst, H., Die histologischen Veränderungen bei der Sklerose der Pulmonalarterie, *Beitr. path. Anat. u. allg. Path.*, 1904, xxxvi, 210.

[17] Aschoff, L., Arteriosklerose, *Beiheft med. Klin.*, 1914, x, 1.

the lungs and increased pressure from the right heart is a sufficiently long time to permit degenerative changes to develop in human pulmonary arteries.

One would think that if cholesterol or its esters participate actively in causing sclerotic lesions, such activity would be plainly demonstrated by the present experiments. That cholesterol is practically inert, that no esters, at least no doubly refracting bodies, have been met with, appears to be one of the striking differences between the arterial lesions of our dogs and human atherosclerosis. This discrepancy is, however, not so great as it would seem. The modern trend of opinion, especially as represented by Aschoff, seems to be that deposits of cholesterol esters are secondary to the mechanical conditions by means of which the intima, and perhaps also the media, undergoes serious structural alterations. Plasma is forced into the intima and the amount of lipoid deposit that takes place depends largely on the cholesterol ester content of that plasma. With this precipitation of lipoids, degeneration begins, the *Kittsubstanz* is demolished, and those processes are initiated that lead to atheroma.

This conception is, however, not as yet a well grounded fact, but rather an hypothesis. It is true that cholesterol and its esters are invariably found in every case of human atheroma and that the extent of the degeneration is approximately proportionate to the quantity of lipoids accumulated within the vascular tissues, but up to date nothing definite is as yet known about the exact conditions that induce the lipoid deposits or of the specific functions of the latter. Perhaps there may be some truth in the theories propounded by Lemoine and Gèrard,[18] according to which the cholesterol acts as an antitoxin and is heaped up in the degenerating vascular tissues in order to neutralize and to assist in transporting the toxic material injected into the blood vessels by the various processes and diseases that are supposed to lead to arteriosclerosis. In this connection it is significant that Klotz[3] finds that the hemolytic properties of sodium oleate are neutralized by an adequate admixture of cholesterol. Some added interest as regards the function of cholesterol in arterio-

[18] Lemoine and Gèrard, in Bertrand, Nature de l'athérome et rôle de la cholestèrine dans sa formation, *Rev. mod. mêd. et Chir.*, 1913, xi, 380.

sclerosis may also be found in recent studies of sclerotic lesions as found in very young persons and babies, especially by Stumpf[19] and Saltykow.[20] The present writer has also examined a relatively large number of infantile aortas, of several fetuses, and also of dogs. He hopes to publish his results in the near future, but it may be stated now that in the majority of the baby aortas, no matter what the cause of death, no matter how old or how young the child, minute ridges and patches slightly elevated above the level of the intima, and of a faint yellowish color, could be found. They were most frequently found in the sinus Valsalvæ, the bulb or the arch, but often in the descending and abdominal aorta and usually in the neighborhood of the orifices of branching vessels. Under the microscope there could regularly be seen a very distinct and typical hyperplasia of the intima, a practically intact media, very minute sudanophil droplets scattered in the interstices between the elements of the intima, and also fairly large phagocytes filled with sudanophil granules. The same phenomena could with great constancy be found also in the aorta of young dogs. In these cases there were no signs of degeneration, nor did there appear to be any tendency towards atheroma, and it must be a subject for further study to ascertain the functions of the cholesterol, and for what reason and by what mechanism a baby 1 day old or a 7 months fetus had small areas of hyperplastic intima and sudanophil phagocytes in the aorta. It seems probable that the distribution and the presumptive action of cholesterol in these cases and that found in the pulmonary arteries of our dogs are similar.

As a result of this brief discussion it is believed that the arterial lesions experimentally obtained in our dogs are essentially identical with those found in the human pulmonary artery, and that for the first time atherosclerosis, very closely analogous to the human, has with certainty been produced experimentally in dogs.

[19] Stumpf, R., Über die Entartungsvorgänge in der Aorta des Kindes und ihre Beziehungen zur Atherosklerose, *Beitr. path. Anat. u. allg. Path.*, 1914, lix, 390.

[20] Saltykow, S., Jungendliche und beginnende Atherosklerose, *Cor.-Bl. Schweiz. Aerzte*, 1915, xlv, 1057, 1089.

SUMMARY.

Cholesterol dissolved in sesame oil and injected regularly for a period of from 7 to 8 months into the jugular vein of four young dogs has caused in each animal larger and smaller nodules protruding to some degree into the lumen of the pulmonary artery and also here and there some diffuse thickenings, the whole closely resembling human arteriosclerosis. These changes commence at the origin of the pulmonary artery immediately behind the semilunar valves. They seem a little more pronounced in those dogs that had the aorta tied. Microscopically the lesions are seen to be primary in the media. They consist of a more or less violent disorganization of the elastic elements and displacement of the muscular tissue. As a consequence wide gaps, apparently filled with plasma, are formed in the media. The entire process is localized principally in the inner third of the media. There is no evidence of fatty degeneration either in the elastic elements or the muscle. Typical sclerotic hyperplasia of the intima is found over some of the prominent median nodulations while over others the intima is normal. Hyperplastic sclerosis of the intima is also found in places where the media is intact or only slightly damaged. Some of the larger branches of the pulmonary artery well within the lung tissue are somewhat dilated and show extensive hyperplasia of the intima over almost normal media. Peculiar small nodules are seen in the media of the two main branches of the pulmonary artery which are found to consist of frank hyperplasia of the elastic and muscular elements arranged in more or less regular concentric circles. The capillaries and smaller and smallest arteries within the lungs are not affected. Cholesterol in phagocytes and in droplets between the tissue elements is present in very small quantities and does not take any active part in the process. The entire process represents in all probability an early stage of arteriosclerosis, a result of purely mechanical stress brought about by the very extensive but more or less intermittent blocking of the pulmonary circulation by the oil. The musculature of the artery appears to be the main force applied to overcome the resistance in the circulation. In no case has hypertrophy of the right heart been found.

The lesions in the pulmonary arteries of the dogs produced experimentally are closely analogous to atherosclerosis of the human pulmonary artery.

EXPLANATION OF PLATES.

PLATE 40.

FIG. 1. Left heart and aorta of a dog whose aorta had been tied, showing sclerotic nodules behind the cusps and ridges in the further course of the vessel.

FIG 2. Extensive sclerosis of the pulmonary artery, possibly some dilatation of the right ventricle.

PLATE 41.

FIG. 3. The widespread disorganization of the media, the large gaps containing fragments of elastic fibers, and faintly stained coagulated material are shown. Muscle cells normal, extensive splitting of elastica interna. No change in the intima. Hematoxylin and Sudan III; Zeiss oc. 4, apochromatic 3 mm.

PLATE 42.

FIG. 4. Frozen section. The disruption of the inner portions of the media and moderate hyperplasia of the intima are shown. The elastica interna is only partially intact. Hematoxylin and Sudan III; Zeiss oc. 4, apochromatic 8 mm.

PLATE 43.

FIG. 5. Paraffin section of a branch of the pulmonary artery within the lung. Typical sclerotic hyperplasia of the intima, some disorganization of the inner portions of the media. To the right of the picture the intima with its endothelium is seen to be perfectly normal. Hematoxylin and eosin; Zeiss oc. 4, apochromatic 16 mm.

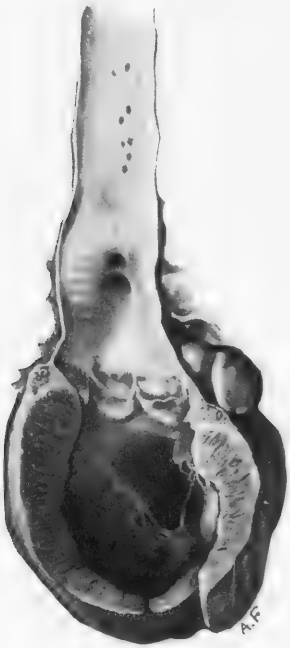

FIG. 1.

FIG. 2.

(Adler: Experimental atherosclerosis.)

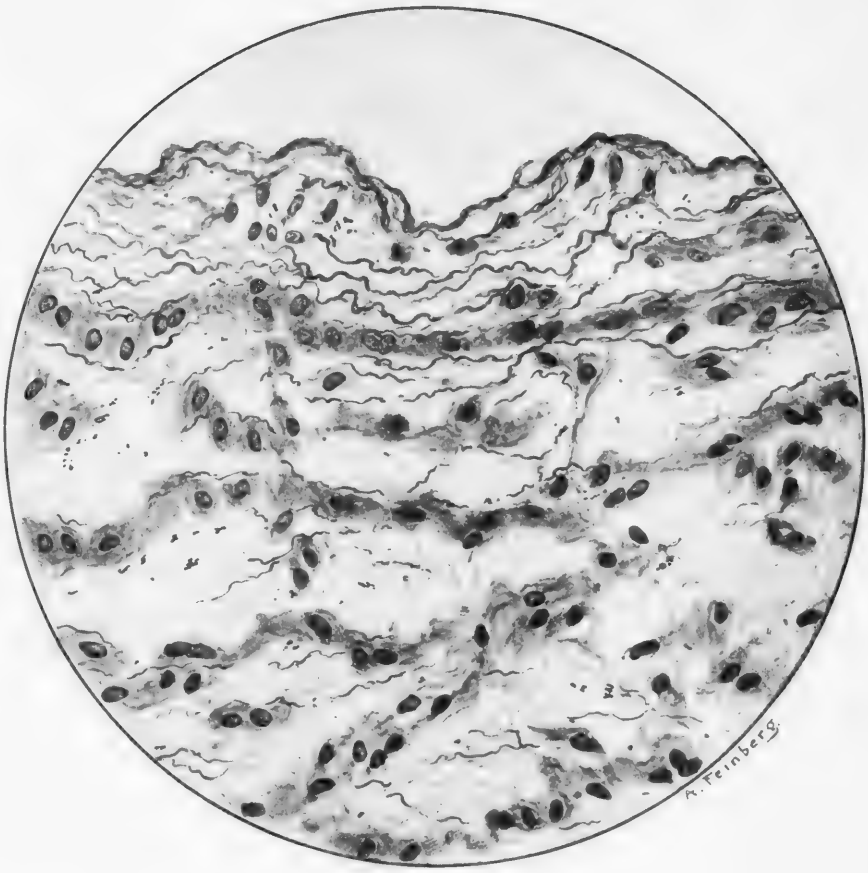

FIG. 3.

(Adler: Experimental atherosclerosis.)

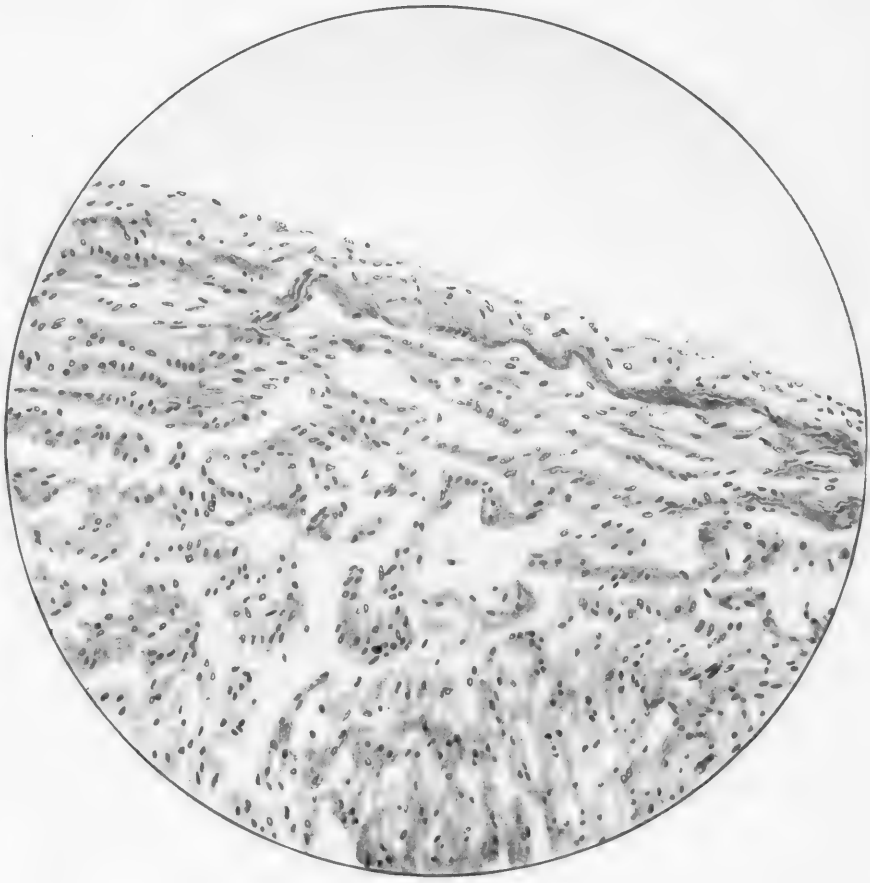

FIG. 4.

(Adler: Experimental atherosclerosis.)

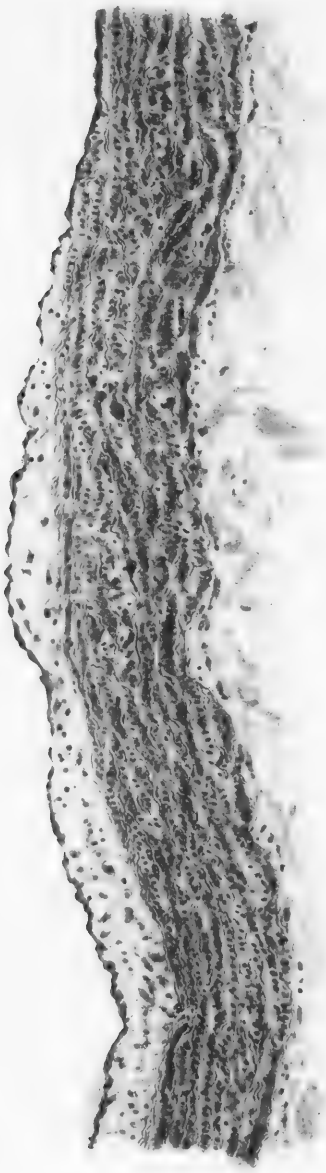

FIG. 5.

(Adler: Experimental atherosclerosis.)

THE PROPHYLACTIC AND THERAPEUTIC PROPERTIES OF THE ANTITOXIN FOR BACILLUS WELCHII.

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In a recent article[1] experiments were given which seemed to establish the following points: (1) under certain defined conditions, a bacteria-free toxic substance can be obtained from cultures of *Bacillus welchii;* (2) this substance possesses the physical properties of an exotoxin and, on animal inoculation, is capable of producing all the essential lesions and effects of infection with the bacilli; (3) animals which have received a number of graded doses of the toxin yield an immune serum which neutralizes *in vitro* all the pathologic effects of the toxin and exhibits power to prevent and control infections with both the spore and vegetative forms of the bacilli. These experimental results appeared to justify the conclusion that *Bacillus welchii* should be classed with *Bacillus diphtheriæ* and *Bacillus tetani* as a toxin-producing organism and that infections with the organism might be successfully combated by means of a specific immune serum. The present paper deals with more extensive and systematic experiments on the preventive and curative powers of the antitoxin.

Prophylaxis against Intoxication.

A series of guinea pigs ranging in weight from 500 to 800 gm. was given a prophylactic dose of antitoxic serum. Each animal received 0.25 cc. of the antitoxin[2] for each 100 gm. of weight, and the susceptibility of the prophylactic series to intravenous injections of the toxin

[1] Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, xxvi, 119.

[2] This antitoxin has now been prepared in the horse. The method employed for producing it will be the subject of a later paper, as well as the question of standardization. At present dosage is given in numbers of cubic centimeters of the serum employed.

was compared with that of normal guinea pigs at different intervals after the immune serum had been given. The following protocols give the results:

Experiment 1. 2 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 1, weight 510 gm., had received 1.5 cc. of the antitoxin subcutaneously. 10 cc. of toxin were injected into the jugular vein. No symptoms had developed 6 hours after the injection, but the guinea pig was found dead 20 hours after receiving the toxin. The autopsy showed lesions characteristic of *B. welchii* toxin.[3]

Guinea Pig 2, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 8 cc. of toxin were injected into the jugular vein. The guinea pig developed no symptoms and was discarded in perfect condition 3 weeks later.

Guinea Pig 3, weight 560 gm., normal control. 0.3 cc. of toxin was injected into the jugular vein. This guinea pig was found dead 16 hours later. Characteristic lesions were found at autopsy.

Experiment 2. 5 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 4, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 4 cc. of toxin were injected into the jugular vein. This animal developed symptoms of intoxication 2 hours after the injection and died at the expiration of 4 hours. The autopsy findings were characteristic.

Guinea Pig 5, weight 510 gm., had received 1.5 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No immediate or delayed symptoms arose, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 6, weight 770 gm., normal control. 0.25 cc. of toxin was injected into the jugular vein. The guinea pig was found dead 16 hours later. The autopsy findings were typical.

Experiment 3. 7 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 7, weight 670 gm., had received 1.7 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms developed, and the guinea pig was normal 3 weeks later.

Guinea Pig 8, weight 600 gm., normal control. 1 cc. of toxin was injected into the jugular vein. The guinea pig died $3\frac{1}{2}$ hours later, and characteristic lesions were present.

Experiment 4. 9 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 9, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms had appeared 6 hours later, but the guinea pig was found dead 24 hours after the toxin was given. The autopsy findings were characteristic of *B. welchii* intoxication.

Guinea Pig 10, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 1.5 cc. of toxin were injected into the jugular vein. No symptoms of intoxication arose, and the guinea pig remained normal.

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[3] Differences in the manner of action of *B. welchii* toxin when administered by intravenous and subcutaneous routes are described in the paper already referred to.[1]

Guinea Pig 11, weight 700 gm., normal control. 0.7 cc. of toxin was given intravenously. Death occurred 4 hours later, and the lesions were typical.

Experiment 5. 12 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 12, weight 640 gm., had received 1.6 cc. of antitoxin subcutaneously. 0.7 cc. of toxin was injected into the jugular vein. No symptoms had arisen 8 hours later. The guinea pig was found dead 22 hours after the toxin was given, and the autopsy findings were typical.

Guinea Pig 13, weight 660 gm., had received 1.7 cc. of antitoxin subcutaneously. 0.5 cc. of toxin was injected into the jugular vein. The guinea pig remained normal.

Guinea Pig 14, weight 600 gm., normal control. 0.28 cc. of toxin was given intravenously. This guinea pig was found dead 20 hours later. The autopsy findings were typical.

Experiment 6. 14 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 15, weight 570 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was injected into the jugular vein. Death occurred 20 hours later. Characteristic toxin lesions were found at autopsy.

Guinea Pig 16, weight 580 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was given intravenously. No symptoms developed, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 17, weight 600 gm., normal control. 0.27 cc. of toxin was injected into the jugular vein. The animal was found dead 20 hours after the toxin was given, and typical lesions were present.

The foregoing experiments show that a passive immunity to *Bacillus welchii* toxin can be conferred on guinea pigs by the administration of the antitoxin. In these instances the immunity persisted for about 2 weeks. The protection from the toxin was at first very pronounced, thirty-two acutely lethal doses being harmless when injected directly into the blood stream (Experiment 1, Guinea Pig 2) 2 days after the antitoxin was given. 5 days after the prophylactic administration of the antitoxin, eight lethal doses were still harmless (Experiment 2, Guinea Pig 5), but sixteen lethal doses killed. The immunity to the toxin gradually decreased and was about exhausted at the end of 2 weeks. At this time some of the prophylactic animals were still somewhat more resistant to the toxin than normal animals, while others manifested no increased resistance (Experiment 6, Guinea Pigs 15, 16, and 17).

The fact that animals can be passively immunized to the toxin may in itself be of considerable practical significance; but when it is remembered that the toxin is a powerful aggressin, preparing the field

for bacterial multiplication, its high significance becomes at once apparent. The next series of experiments further emphasizes the importance of the part played by the toxin in infection by the bacilli and shows conclusively that large numbers of highly virulent bacilli are practically harmless when deprived of their toxin.

Prophylaxis against Infection.

A series of guinea pigs, ranging in weight from 250 to 350 gm., was given subcutaneously on the inner aspect of one hind leg 1 cc. of antitoxin per 100 gm. of weight. The resistance of the prophylactic series to infection with fresh cultures of the virulent bacilli was compared with that of normal guinea pigs of the same size. The protected guinea pigs were infected subcutaneously in the leg opposite to the one in which they had received the antitoxin. The quantity of culture given the individual animals was calculated on the basis of body weight, a certain fraction of a cubic centimeter being given for each 100 gm. of weight. The following protocols illustrate the results:

Experiment 7. 24 Hours after the Prophylactic Dose of Antitoxin.—Guinea Pig 18, weight 280 gm., had received 2.8 cc. of antitoxin. 0.28 cc. of culture was given subcutaneously. 7 hours later the infected leg was moderately swollen and stiff; no local crepitation and no symptoms of general intoxication. 24 hours after the inoculation the local swelling had almost disappeared, and the guinea pig was apparently well.

Guinea Pig 19, weight 280 gm., had received 2.8 cc. of antitoxin and was given 0.5 cc. of culture. 7 hours later the infected leg was swollen and stiff; crepitation could not be elicited, and there was no general intoxication. The next day the swelling and stiffness were subsiding, the guinea pig was active, and no extension of the infection arose.

Guinea Pig 20, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.9 cc. of culture. The results were identical with those of the two preceding animals.

Guinea Pig 21, weight 310 gm., normal control. 0.006 cc. of culture was given subcutaneously. This guinea pig was found dead 22 hours after it was inoculated. Autopsy revealed edema, gas, and disorganization of tissue, lesions typical of *B. welchii* infection.

In Experiment 7 it is shown that 50, 83, and 150 lethal doses of culture respectively failed to infect the guinea pigs which had re-

ceived a protective injection of antitoxin 24 hours previously. As 150 lethal doses was the largest quantity of culture given the height of the resistance to infection was not accurately determined.

Experiment 8. 3 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 22, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.4 cc. of culture. No symptoms developed aside from local swelling and stiffness which rapidly subsided. No evidence of multiplication of the bacilli.

Guinea Pig 23, weight 310 gm., had received 3.1 cc. of antitoxin. 0.6 cc. of culture was injected into the opposite leg. The symptoms and results were the same as in the preceding animal.

Guinea Pig 24, weight 300 gm., normal control. 0.004 cc. of culture was given subcutaneously. The animal died 22 hours later. Lesions of *B. welchii* infection were present at autopsy.

In this experiment the limit of the resistance to infection was again not reached, although one of the guinea pigs also received 150 lethal doses of the culture.

Experiment 9. 5 Days after the Antitoxin.—Guinea Pig 25, weight 260 gm., had received 2.6 cc. of antitoxin. 1.5 cc. of culture were injected into the opposite leg. Infection did not develop, and the local swelling and stiffness which always follow these inoculations rapidly subsided.

Guinea Pig 26, weight 280 gm., had received 2.8 cc. of antitoxin. 2 cc. of culture were given. Infection developed, and the guinea pig died 30 hours after inoculation. Lesions typical of *B. welchii* infection were present at autopsy.

Guinea Pig 27, weight 290 gm., normal control. 0.005 cc. of culture was injected subcutaneously. The guinea pig was found dead 20 hours later and showed typical lesions.

This experiment indicates that a protected guinea pig could resist 300 but not 400 lethal doses of the culture 5 days after the antitoxin had been given.

Experiment 10. 8 Days after the Antitoxin.—Guinea Pig 28, weight 260 gm., had received 2.6 cc. of antitoxin. 0.3 cc. of culture was given subcutaneously. The usual local swelling and stiffness followed, but no infection arose.

Guinea Pig 29, weight 280 gm., had received 2.8 cc. of antitoxin subcutaneously. 0.5 cc. of culture caused a fatal infection, with characteristic lesions.

Guinea Pig 30, weight 275 gm., normal control. 0.005 cc. of culture was given subcutaneously. Infection developed, and the guinea pig died 23 hours after inoculation.

This experiment shows that 60 lethal doses of culture failed to infect 8 days after the antitoxin had been administered. The resistance of the protected guinea pigs was tested again on the 11th day, and twenty lethal doses were not infectious. On the 14th and 15th days, however, the antitoxin-treated guinea pigs proved susceptible to infection, but whether to the same degree as the normal controls the experiment does not indicate. We may, however, assume that the antitoxin becomes greatly reduced in quantity from the 12th to 13th day after its administration.

Therapeutic Property of the Antitoxin.

We may now consider the effects which the antitoxin exerts upon established infection with *Bacillus welchii*. For this purpose guinea pigs weighing from 500 to 600 gm. were used, smaller ones being so susceptible to the infection that there is little time afforded for treatment. The amount of a standard culture that would infect and kill all of a series of guinea pigs between 30 and 48 hours was first determined. The infecting dose was calculated on the basis of body weight, and it was found that about 0.035 cc. of culture per 100 gm. of weight was effective within the time limit mentioned in all the animals inoculated ranging in weight from 500 to 600 gm. The procedure was to infect a number of animals at one time with the same culture and allow 24 hours to elapse before beginning treatment. At this time the condition of the animals was noted, especially the extent of the local lesions and the symptoms of general intoxication. One of the animals was etherized and autopsied, and to one or more the antitoxin was administered, while the others were left as untreated controls. Illustrative protocols follow:

Experiment 11.—Guinea Pig 31, weight 510 gm. 0.18 cc. of culture was injected into the muscles on the inner aspect of the hind leg. 24 hours after inoculation, a gaseous phlegmon involving the infected leg and the adjacent abdominal wall had developed, crepitation was readily elicited, and the guinea pig exhibited symptoms of severe intoxication—rough coat, rapid respiration, and drowsiness. 3 cc. of antitoxin were injected into the jugular vein. The next day the local lesion was subsiding, crepitation could not be elicited, the toxic symptoms had disappeared, and the guinea pig ate greedily. 3 cc. of antitoxin were given sub-

cutaneously to prevent a recrudescence of the infection. The general condition improved from day to day, while the infected leg became gangrenous, sloughed, and was infected with pyogenic organisms. Healing finally occurred, but the scar tissue caused deformity of the leg.

Guinea Pig 32, weight 560 gm. 0.2 cc. of culture was injected into the muscle on the inner aspect of the hind leg. 24 hours after inoculation there were swelling and edema, crepitation, and intoxication. The animal was not treated, and it died exactly 48 hours after inoculation. Autopsy revealed the characteristic lesions of *B. welchii* infection.

Guinea Pig 33, weight 580 gm. 0.2 cc. of culture was injected in the same manner as in Guinea Pigs 31 and 32. 24 hours after inoculation, the guinea pig was etherized and autopsied. There was a large gaseous phlegmon involving the infected leg and adjacent abdominal wall. The muscles of this region were pulpified and laden with bacilli. The opposite groin contained a gelatinous, serosanguineous exudate. The muscles of the abdominal wall and diaphragm were of pinkish hue. The lungs were pink and edematous.

Guinea Pig 34, weight 605 gm., was inoculated with 0.21 cc. of culture. This animal served as an untreated control and died at the end of 40 hours of typical *B. welchii* infection.

Experiment 11 was repeated a number of times, and it was found that the infection could be regularly arrested by the antitoxin after it was well established and extensive destruction of the tissues in the region of the infection had already taken place. Symptoms of severe general intoxication were always present at this stage of the disease. The effect of the antitoxin was often noticeable within from 30 minutes to 1 hour after its administration. The treated guinea pigs would become more active, their coats smooth, and they would be attracted by food, while the controls remained crouched in a corner, with heads down and coats rough, and could not be induced to eat.

DISCUSSION.

The experimental results here reported with the preventive and therapeutic applications of the antitoxin are highly suggestive. They derive significance from the fact that *Bacillus welchii* infections in guinea pigs and other susceptible animals[4] are comparable with in-

[4] Protection and curative experiments have been carried out also with the more highly susceptible pigeon. They confirm the results with guinea pigs with certain variations due to the difference in species.

fections with this organism in man. The experimental infections in the guinea pig differ, however, from the natural infection in man in two important points: (1) man possesses a higher natural resistance to infection; (2) the guinea pigs were infected with fresh virulent cultures, while man must, in the great majority of instances, derive infection from spores. Moreover, the protected guinea pigs were given many lethal doses of the living cultures. Such massive inoculations do not occur in man. It may therefore be safely predicted that man will not develop the infection as long as his body fluids and tissues contain adequate quantities of the antitoxin.

The possibilities of this passive serum protection has natural limits of time, depending upon the rapidity of elimination of the foreign serum. The experimental data presented in this paper, which agree with the experience with antidiphtheritic and antitetanic antitoxins, indicate that, in all probability, a passive immunity to *Bacillus welchii* infection of at least 2 weeks' duration can be conferred upon man by a single injection of the antitoxin. This immunity will be sufficient in the majority of instances, since only sporadic cases of *Bacillus welchii* infection arise later than the 10th day after injury, and the greater number occurs within 48 hours of that time.

In the light of the results obtained in treating the infection in guinea pigs, it is reasonable to hope that the antitoxin will be of value also as a therapeutic agent. The indications are that early infectious cases can be readily arrested and the more advanced and severe ones ameliorated, if not wholly checked, so that surgical interference may be resorted to with greater probability of effectiveness.

The antitoxin in man should be administered intravenously and probably locally, about the wound, as well.

SUMMARY.

1. It has been possible to confer on guinea pigs a passive immunity of about 2 weeks' duration to *Bacillus welchii* toxin through a protective administration of the antitoxin.

2. Guinea pigs which had received a prophylactic dose of *Bacillus welchii* antitoxin exhibited pronounced resistance to infection with the virulent bacilli for a period of 12 days.

3. Established infections in guinea pigs with *Bacillus welchii* have been arrested and controlled by treatment with the antitoxin.

4. The opinion has been expressed that it will be possible to prevent *Bacillus welchii* infection in man through the prophylactic use of the antitoxin and developed cases of the infection may be controlled by therapeutic injections of the same agent.